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Award Number: DAMD17-00-1-0528

TITLE: Prostate Cancer Cell Growth: Role of Neurotensin in Mediating Effect of Dietary Fat and Mechanism of Action

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REPORT DATE: October 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Final (1 Oct 2000 - 30 Sep 2004)

4. TITLE AND SUBTITLE
Prostate Cancer Cell Growth: Role of Neurotensin in Mediating Effect of Dietary Fat and Mechanism of Action

5. FUNDING NUMBERS
DAMD17-00-1-0528

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Aims: (a) study mechanism of neurotensin(NT)-enhanced prostate cancer(PC)growth; (b)test if NT released from gut contributes to the PC-growth effect of fatty diets. In PC3 cells, NT transactivated EGF receptor (EGFR), liberating HB-EGF from cell-surface by a protease-dependent and PKC-dependent mechanism. Activation of EGFR led to activation of MAP-kinase, PI3-kinase, AKT and p70-S6-kinase, and in 24 hrs to enhanced DNA synthesis. NT and EGF stimulated cellular release 3H-AA. Inhibitors of Lipoxygenase (LOX) blocked basal and NT-induced DNA synthesis, suggesting that EGF and NT acted by way of PLA2, 5-LOX and 12-LOX (present by western blot). NT receptor function was modulated by antioxidants and calcium channel blockers, which indirectly blocked inositol phosphate formation. In the presence of a Gs stimulus, NT enhanced cAMP production, giving an inhibition of DNA synthesis. Thus, NT effects involve EGFR, protein kinases, LOXs and adenylyl cyclases, and its effects depend on the hormonal milieu. An NT-knockout-nude mouse strain was developed and PC3 cell xenografts were found to grow faster in NT(++) mice than in NT(--) mice given a high fat diet. Our results suggest that NT released during fat ingestion stimulates PC growth via effects involving EGFR, protein kinases, LOXs and calcium channels.

14. SUBJECT TERMS
Cell growth, neuroendocrine peptide, cell signaling, neurotensin, dietary effects, signal transduction, receptor binding

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

The overall goal of this work is to examine the involvement of the intestinal hormone neurotensin (NT) in the regulation of prostate cancer (PC) growth. The initial hypothesis under study is that NT, which is released from the intestine by ingestion of fatty meals, contributes to the enhanced growth of PC cells in animals consuming high fat food, and that the effects of NT may explain the worldwide association between high fat intake and growth / incidence of PC in humans. Since NT has been shown to exert important effects on growth of both androgen-independent (PC3) and androgen-dependent (LNCaP) human PC cells *in vitro*, a key goal is to understand the mechanism(s) involved in its growth promoting effect(s) in these two cell lines (1).

To examine the mechanism(s) involved in NT-induced growth promotion, we have performed experiments using PC3 and LNCaP cells in tissue culture, and we studied the interaction of NT with major growth factor pathways. We have characterized NT binding to the cells and examined its regulation. Of particular interest are the effects of NT on the EGF pathway. Attention has been given to growth regulation by the MAPK-, Akt- and CDK-pathways. We have identified NT inputs into the phosphorylation of the key enzymes in these pathways and we have investigated relationships to inositol phosphate (IP) formation, Ca^{2+}-release, Ca^{2+}-influx, PKC-activation and cAMP formation (2, 3).

To examine the contribution of NT to PC tumor growth *in vivo*, we have utilized an animal model involving PC3 cells xenografted into nude mice. We also developed an NT knockout-nude mouse to address this issue. Some animals are maintained on high and low fat diets and the effects of NT antagonist SR48692 on PC tumor growth are also being measured.

BODY:

Task #1 - To compare the effects of NT antagonist on the growth of PC3 and LNCaP cells in nude mice (months 1-30).

Initial studies were performed using PC3 cells and the data showed that daily injections of NT antagonist SR48692 slowed the growth rate of PC3 tumors in nude mice. However, we were not satisfied with the test system and tried to improve the method. We described our quandary as to whether xenografting was best achieved by the injection of cancer cells or the implantation of cubes of tumor tissue using a trocar syringe. We received instruction from another cancer laboratory where the cell injection method was optimized to avoid the difficulty of multiple tumors. We applied their technique to perform an experiment to compare tumor growth in NT knockout mice and control nude mice fed high fat food.

NT knockout mice and controls were inoculated with tumor cells that were settled as a mass into the end of a 26-gauge needle. By injecting them as a clump, we intended to favor the formation of a single tumor. Although single tumors were

obtained, it took 2 months before they were evident and the experiment lasted 6 months. On the positive side, the results were encouraging. Tumor growth rates were less at all times for the NT knockout mice than for the controls. Although the measurements did not achieve statistical significance, there was a clear trend towards slower growth in the NT knockout mice.

Since the slow tumor development greatly increased the cost of animal care, we used tumor cubes in the following experiments. We learned the trocar syringe method and found that it could be done with minimal stress and injury. Donor mice were chosen from the earlier experiment and we implanted 2 tumor cubes (3 mm^3) at separate sites in 12 nude mice (NT(++) strain) and 12 NT knockout nude mice (NT(--) strain). Tumors developed quicker with this method and the variability in tumor size was less. Tumor growth rates were less again in the NT knockout animals and the level of significance was improved.

Having mastered the technique, we undertook a final experiment, implanting tumor cubes in 12 NT(++) mice and 9 NT(--) mice. The results for this experiment indicated that tumor growth rates were indeed significantly slower in NT knockout mice. The log of tumor volume was related linearly to days of tumor growth. The increment in tumor volume measured 30 days after the tumor reached 50mm^3 was 1567±183 mm^3 (NT++) and 599±120 mm^3 (NT--), which differed significantly ($p<0.01$). When adequate funding is available, we intend to continue these studies which at this time are very encouraging.

Task #2 - Perform dietary studies to correlate NT levels with fat intake and cancer cell growth (months 18-36).

We had insufficient time to perform these studies.

Task #3 - To develop NT knock-out / nude mouse strain from our NT knock-out and nu/nu purchased from Jackson Labs (months 1-24).

As reported earlier, we have succeeded in accomplishing this task.

Task #4 - Test effect of fat on cancer growth in NT-knockout/nude mice (months 16-36).

The studies described in Task #1 showed that tumor growth was slower in NT knockout mice as compared to control mice that were fed a high fat diet. There was insufficient time to examine the effects of high versus low fat diets.

Task #5 - Examine effects of NT on arachidonic acid metabolism and on lipoxygenase expression in prostate cancer cells (months 1-12).

We have extended the observations reported earlier regarding the effects of NT on arachidonic acid (AA) metabolism. NT and EGF were previously shown to liberate

[3H]-AA from labeled PC3 cells and the effect of NT was inhibited by PKC inhibitor (staurosporine), PI3-kinase inhibitor (LY294002 and wortmannin), MEK inhibitor (U0126), calcium channel blocker (nifedipine) and EGFR-tyrosine kinase inhibitor (AG1478). These results supported the idea that NT activated MAP-kinase and that this enzyme, which is known to activate PLA2, liberates AA from the phospholipid pool. Now, we have shown by western blotting that PC3 cells express cPLA2 but not sPLA2 and iPLA2. In addition, the cells expressed 5-lipoxygense and 12-lipoxygenase but not 15-lipoxygenase. These results are consistent with the pattern of ^{3}H-AA metabolites detected after NT treatments.

Task #6 - Test NT for ability to alter growth responses to EGF in PC3 cells (months 13-24).

As reported earlier, we have completed most of this task. In additional studies, we have now shown that NT stimulates PKC-dependent PKD activation in PC3 cells. EGF also activated PKD and our results thus far are consistent with the idea that NT acts by transactivating the EGF-receptor. It may be that an AA-metabolite mediates the activation of PKC.

Other work shows that NT-induced EGFR/AKT/MAPK activation and DNA synthesis was attenuated by metallo-endopeptidase inhibitor phosphoramidon. This is in agreement with the involvement of HB-EGF in the NT pathway. Using western blotting, we also showed that NT activates p70 S6-kinase which is downstream of AKT and MAPK and a reliable indicator of mitogenesis.

In the course of investigating the Ca^{2+} dependence of NT signaling, we also discovered that L-type Ca^{2+}-channel blockers enhanced NT binding and inhibited NT-induced IP formation. This is discussed at length under "task #7".

Task #7 - Test NT for ability to activate MAP-kinases and to induce indicators of mitogenic stimulation (months 25-36).

In the last report, we indicated that NT dose-responsively (0.1 to 30 nM) enhanced the activation (phosphorylation) of EGFR, ERK1/2 and Akt, but it did not affect JNK/SAPK and p38 MAPK. The NT-induced transactivation of EGFR and the enhancement in DNA synthesis that followed were blocked by heparin (10μg/ml) but were unaffected by neutralizing anti-EGF (10μg/ml), indicating the involvement of HB-EGF instead of EGF itself. Since HB-EGF can be liberated from the cell surface by PKC-dependent steps, we examined the involvement of PKC. The EGFR transactivation, the ERK activation and the stimulation of DNA synthesis in response to NT were all blocked by PKC inhibitor (staurosporine) and by downregulation of PKC, indicating a mediator role for PKC. Our model at this time was that NT interacated with its Gq-coupled receptor to stimulate PLC-mediated formation of inositol phosphates (which elevated Ca^{2+}) and DAG (which activated PKC). PKC then releases HB-EGF, activating the EGFR-MAP-kinase cascade and stimulating DNA synthesis. This topic

was the subject of a manuscript we published in *Regulatory Peptides* 120:155-166 (2004).

Although NT-induced EGFR transactivation and ERK phosphorylation were not blocked by intracellular Ca^{2+}-chelator (BAPTA-AM), nor by extracellular chelator (EGTA), these effects were inhibited by Ca^{2+}-channel blockers (e.g., nifedipine). To better understand the mechanism(s) involved, we screened numerous Ca^{2+}-channel blockers for effects on NT-receptor binding and on NT-induced inositol phosphate (IP) formation. We hypothesized that Ca^{2+}-channels might somehow interact directly with NT-receptors. Ca^{2+}-channel blockers from 7 chemical categories altered NT binding and NT-induced IP formation. 1,4-dihydropyridines, which are specific for L-type Ca^{2+}-channels, were the most effective agents; however, their effects correlated best to inhibition of store-operated Ca^{2+}-channels (SOCC). Since NT is known to empty internal Ca^{2+}-stores, we hypothesized that it stimulated Ca^{2+}-influx through SOCC. We then showed that NT caused an influx of $^{45}Ca^{2+}$ that was blocked by nifedipine. The results of further studies were in keeping with the idea that IP formation in response to NT involved both PLCβ and PLCδ. NT initially stimulated PLCβ which (via IP3) emptied internal Ca^{2+} stores and stimulated SOCC-mediated Ca^{2+}-influx. The Ca^{2+}-influx then activated PLCδ, generating further IP. This is the subject of a manuscript we published in *Journal of Pharmacology and Experimental Therapeutics* 307:640-650 (2003).

1,4-dihydropyridines also exerted major effects on NT-receptor binding (3-fold enhancement) which we are still sorting out. Our current findings are in keeping with the idea that this effect results from an indirect antioxidative action. These drugs do not alter NT-receptor binding to cellular membranes, indicating a requirement for cellular metabolism or architecture. Enhanced binding was associated with decreased function. Interestingly, the EGF-receptor displays a similar behavior in that Tyr-kinase inhibitors block function while greatly enhancing binding. For NT-receptor, there is a 3-fold shift in receptor affinity without a change in receptor number or receptor internalization. The effects of a series of 1,4-dihydropyridines correlates to their ability to react with superoxide anion. Furthermore, some polyphenolic antioxidants could mimic these effects. This is the subject of a manuscript we published in *Journal of Pharmacology and Experimental Therapeutics* 309:92-101 (2004).

To follow this up, we are currently investigating the effects of Ca^{2+}-channel blockers on cell growth induced by NT, EGF and serum. We are pursuing this line of research since it is likely to reveal key steps in the NT-signaling cascade. One hypothesis, for example, is that NT-receptor activates NADH-oxidase in the plasma membrane, generating reactive oxygen species (ROS). Since ROS (e.g., superoxide) can transactivate EGF-receptor, it is possible that 1,4-dihydropyridines inhibit this step by scavenging ROS. ROS can also activate certain isoforms of PKC, and PKC can activate the MAP-kinase pathway. Since NT-receptor binding is desensitized by PKC, this provides a feedback loop that could explain how NT binding could be increased in the face of inhibited effects. There is precedence for the involvement of NADH-oxidase in growth promotion and other actions of angiotensin-II on vascular cells in culture.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed NT-knockout/nude mouse strain.
- Demonstrated slower tumor growth in NT knockout mice as compared to controls.
- Demonstrated that NT stimulated DNA synthesis in PC3 and LNCaP cells at near physiologic concentrations (0.1 to 1 nM range).
- Demonstrated that NT transactivated EGFR in PC3 and in LNCaP cells.
- Demonstrated that formation of HB-EGF in response to NT led to the activation of EGFR.
- Demonstrated that NT activated ERK in PC3 and in LNCaP cells.
- Demonstrated that NT activated AKT (PI3-kinase) in PC3 cells.
- Demonstrated that NT-induced activation of EGFR, ERK and AKT in PC3 cells was Ca^{2+}-independent, PKC-dependent and contact inhibited.
- Demonstrated that cAMP inhibited NT-induced activation of ERK, and that NT could enhance cAMP formation in response to Gs-stimuli.
- Demonstrated ^{125}I-NT binding to PC3 cells and the presence of NTR1 by western blotting. Related the binding Kd to the EC50 for IP-formation and to activation of EGFR and ERK.
- Demonstrated that LNCaP expressed less NTR1 by western blotting and showed an inability to bind ^{125}I-NT and to respond to NT by increasing IP-formation. However, LNCaP gave growth responses to NT.

NEW ACCOMPLISHMENTS THIS PERIOD:

- Measured slower tumor growth rates in NT knockout-nude mice as compared to controls.
- Demonstrated NT effects on AA-metabolism involving LOX-pathway are important for growth promotion.
- Demonstrated Effects of Ca^{2+}-channel blockers on NT-receptor binding and IP formation.
- Demonstrated antioxidative mechanism for regulation of NT-binding and IP formation.
- Showed PC3 cell expression of cPLA2, 5-LOX and 12-LOX.
- Showed PKC-dependent PKD activation by NT and by EGF.
- Showed p70 S6-kinase activation by NT.
- Showed metallo-protease involvement in NT-induced EGFR activation.

REPORTABLE OUTCOMES:

Abstracts have been written and presented at meetings:

1. S. Hassan and R.E. Carraway. Involvement of MAP-kinase, PI3-kinase and EGF receptor in the Stimulatory Effect of Neurotensin on DNA-replication in PC3 Cells. Reg Pep 2002 Meeting, Boston MA, Aug 31 - Sept 3,2002.

2. R.E. Carraway, S. Hassan, P.R. Dobner. Positive and Negative Effects of Neurotensin on DNA synthesis in PC3 cells Mediated by EGF receptor and Adenylyl Cyclase, respectively. 9th Prouts Prostate Cancer Meeting, Nov 7-10, 2002.

3. S. Hassan and R.E. Carraway. Signaling mechanisms involved in the acativation of arachidonic acid metabolism in PC3 cell growth by neurotensin and its interaction with epidermal growth factor receptor. Summer Neuropeptide Conference, June 8-12, 2003. *Neuropeptides* 37(2003) 159-199.

Manuscripts have been published or are in preparation:

1. S. Hassan, P.R. Dobner and R.E. Carraway. Involvement of MAP-kinase, PI3-kinase and EGF-receptor in the Stimulatory Effect of Neurotensin on DNA Synthesis in PC3 Cells. *Regul. Peptides,* 120 (2004) 155-166.

2. R.E. Carraway, X. Gui and D.E. Cochrane. Ca^{2+}-channel Blockers Enhance Neurotensin (NT) Binding and Inhibit NT-induced Inositol Phosphate Formation in Prostate Cancer PC3 Cells. *J Pharmacol Exp Therap*, 307 (2003) 640-650.

3. R.E. Carraway, S. Hassan and D.E. Cochrane. Polyphenolic Antioxidants Mimic the Effects of 1,4 Dihydropyridines on Neurotensin Receptor Function in PC3 Cells. *J Pharmacol Exp Therap,* 309 (2004) 92-101.

4. R.E. Carraway, S. Hassan and D.E. Cochrane. Effects of Lipoxygenase Pathway Blockers on Neurotensin Receptor Binding and Function in Prostate Cancer PC3 Cells. Submitted to *J. Biol Chem.*(2004).

5. R.E. Carraway, M. Sanderson and D.E. Cochrane. Role of Ca^{2+} and PKC in the cAMP-enhancing Effect of Neurotensin in PC3 cells. *in preparation.*

6. S. Hassan and R.E. Carraway. Role of Arachidonic Acid Metabolism in PC3 Cell Growth Responses to EGF and Neurotensin. *in preparation.*

7. R.E. Carraway and S. Hassan. Role of PKC in ^{125}I-neurotensin (NT) Binding and Biologic Effects Leading to Growth of Prostate Cancer PC3 cells. *in preparation*

CONCLUSIONS:

The importance of the completed work is in regards to the mechanism by which NT enhances the growth of prostate PC3 and LNCaP cells in culture. Our work indicates that in PC3 cells, NT binds to NTR1, transactivating EGFR by liberating HB-EGF from the cell-surface by a metallo-protease-dependent and PKC-dependent mechanism. In LNCaP, the mechanism is PKC-independent and a different receptor may be involved. Temporally associated with activation of EGFR is an activation of MAP-kinase, PI3-kinase and AKT, which is followed a day later by a stimulation of DNA synthesis. These responses depend on the hormonal milieu. In the presence of a Gs-stimulus, NT can enhance cAMP formation, resulting in an inhibition of DNA synthesis. Since we found that LOX inhibitors can reduce basal and NT-stimulated DNA replication, it seems likely that NT & EGF both act by way of PLA2, 5-LOX and 12-LOX. Thus, growth enhancement by NT & EGF involves a coordinate enhancement of LOX(s) and protein kinase(s), and the growth response can be influenced by the activity of adenylyl cyclase(s).

REFERENCES:

1. Seethalakshmi L, Mitra S, Dobner PR, Menon M, Carraway RE. 1997. Neurotensin receptor expression in prostate cell line and growth effect of NT at physiologic concentrations. The Prostate 31:183-192.

2. Carraway RE, Mitra SP. 1998. Neurotensin enhances agonist-induced cAMP accumulation in PC3 cells via Ca2+-dependent adenylyl cyclase(s). Mol. Cell. Endocrinol. 144:47-57.

3. Mitra SP, Carraway RE. 1999. Synergistic effects of neurotensin and β-adrenergic agonist on cAMP accumulation and DNA synthesis in prostate cancer PC3 cells. Biochem. Pharmacol. 57:1391-1397.

0022-3565/03/3072-640–650$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 307:640–650, 2003

Vol. 307, No. 2
52688/1103001
Printed in U.S.A.

Ca^{2+} Channel Blockers Enhance Neurotensin (NT) Binding and Inhibit NT-Induced Inositol Phosphate Formation in Prostate Cancer PC3 Cells

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Received April 4, 2003; accepted July 8, 2003

ABSTRACT

Neurotensin (NT) stimulates Ca^{2+} release and Ca^{2+} influx in many cells. Its contractile effects in smooth muscle are inhibited by removal of Ca^{2+} and by Ca^{2+} channel blockers (CCBs). To better understand NT signaling in prostate cancer PC3 cells, blockers of voltage-gated and store-operated Ca^{2+} channels (VGCC and SOCC) were tested for effects on NT-binding and signaling. Eight chemical types of agents, including VGCC-blocker nifedipine and SOCC-blocker SKF-96365 (1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl]-1*H*-imidazole), enhanced cellular NT binding up to 3-fold, while inhibiting (by ≅70%) NT-induced inositol phosphate (IP) formation. The ability to enhance NT binding correlated with the ability to inhibit NT-induced IP formation, and both effects were relatively specific for NT. Although cellular binding for β_2-adrenergic, V_{1a}-vasopressin, and epidermal growth factor receptors was not enhanced by these drugs, bombesin receptor binding was increased ≅19% and bombesin-induced IP formation was inhibited ≅15%. One difference was that the effect on NT binding was Ca^{2+}-independent, whereas the effect on IP formation was Ca^{2+}-dependent (in part). The Ca^{2+}-dependent part of the IP response seemed to involve SOCC-mediated Ca^{2+} influx to activate phospholipase C (PLC)δ, while the Ca^{2+}-independent part probably involved PLCβ. Photoaffinity labeling of the NT receptor NTR1 was enhanced in CCB-treated cells. NTR1 affinity was increased but NTR1 number and internalization were unchanged. Since CCBs did not alter NT binding to isolated cell membranes, the effects in live cells were indirect. These results suggest that CCBs exert two effects: 1) they inhibit NT-induced IP formation, perhaps by preventing Ca^{2+} influx-dependent activation of PLCδ; and 2) they enhance NTR1 affinity by an unexplained Ca^{2+}-independent mechanism.

Neurotensin (NT), a peptide found primarily in brain and intestine, exerts many effects (Ferris, 1989; Rostene and Alexander, 1997) by activating type 1, G-protein-linked NT receptor NTR1 (Vincent et al., 1999). NTR1 is present on excitable cells (neuroendocrine, smooth muscle) and nonexcitable cells (epithelial, fibroblast) where it activates multiple signaling pathways (Hermans and Maloteaux, 1998). NTR1 is coupled to $G_{q/11}$ since NT stimulates phosphatidylinositol-specific phospholipase C (PLC)-mediated formation of inositol phosphate (IP) and release of intracellular Ca^{2+}. NT also induces Ca^{2+} influx into excitable (Trudeau, 2000) and nonexcitable (Gailly, 1998) cells.

Some actions of NT depend on extracellular [Ca^{2+}] and are inhibited by Ca^{2+} channel blockers (CCBs). Based on the effects of 1,4-dihydropyridines (DHPs) such as nifedipine (NIF), Donoso et al. (1986) and Mule and Serio (1997) suggested that NT-induced intestinal contraction involved Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC). However, in some systems, VGCC current is inhibited by NT (Belmeguenai et al., 2002), and DHPs inhibit NT effects independently of Ca^{2+} (Golba et al., 1995). These contradictory findings led us to investigate the effects of CCBs on NT

This work was supported by Department of Defense (DOD) Grant DAMD17-00-1-0528 and by National Institutes of Health (NIH) Center Grant 5P30-DK32520, although the opinions expressed in the manuscript are not necessarily those of the DOD or the NIH. Part of this material was presented as Abstract P3-576 at the 81st Annual Meeting of the Endocrine Society in June, 1999.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

DOI: 10.1124/jpet.103.052688.

ABBREVIATIONS: NT, neurotensin; NTR1, type 1 NT receptor; PLC, phospholipase C; IP, inositol phosphate; CCBs, Ca^{2+} channel blockers; DHP, dihydropyridine; NIF, nifedipine; VGCC, voltage-gated Ca^{2+} channel; SOCC; store-operated calcium channel; HOLVA, des-Gly-[Phaa^1, D-Tyr(Et)^2,Lys^6,Arg^8]-vasopressin; SKF-96365, 1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl]-1*H*-imidazole; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonylfluoride; EGFR, EGF receptor; GPCR, G-protein-coupled receptor; SR48692, {2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]tricyclo(3.3.1.1.^{3,7})decan-2-carboxylic acid}; AG-1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; PD-153035, 4-[(3-bromophenyl)amino]6,7-dimethoxyquinazoline.

binding and signaling in prostate cancer PC3 cells, which express functional NTR1 (Seethalakshmi et al., 1997). We hypothesized that CCBs could exert effects at multiple levels of the signaling pathway.

Our studies in PC3 cells indicate that NTR1 is linked to $G_{q/11}$ and that stimulation by NT activates PLC, enhances IP formation, and elevates cellular [Ca^{2+}]. This signaling pathway contributes to the regulation of cellular growth by NT (Seethalakshmi et al., 1997) and is linked to a conditional activation of adenylyl cyclase (Carraway and Mitra, 1998). Ca^{2+} is required for NT to stimulate DNA synthesis and to enhance cAMP formation, and these effects are inhibited by the VGCC blocker NIF (R. E. Carraway, unpublished results). Although this suggests that Ca^{2+} influx participates in NT signaling, the roles of Ca^{2+} channels, Na^+/Ca^{2+} exchange, and Ca^{2+} pumps are not defined. The inhibitory effects of NIF implicate VGCC in NT signaling, but this must be questioned since PC3 cells are epithelial and nonexcitable (Putney and Bird, 1993). Another process, which occurs in excitable and nonexcitable cells (Parekh, 2003) subsequent to Ca^{2+} store emptying (Putney, 1999), is capacitative Ca^{2+} entry through store-operated Ca^{2+} channels (SOCC). Given that NT stimulates capacitative Ca^{2+} entry in Chinese hamster ovary cells (Gailly, 1998) and that NT elevates cellular [Ca^{2+}] in PC3 cells (R. E.Carraway, unpublished results), it seems likely that NT stimulates SOCC-mediated Ca^{2+} influx in PC3 cells. Thus, additional studies are required to determine whether NT induces Ca^{2+} influx and to define the channels and mechanisms involved.

Our ability to distinguish mechanisms of Ca^{2+} entry depends largely on the selectivity of CCBs (Harper and Daly, 1999; Triggle, 1999). Based on its possible relevance to NT signaling as discussed above, we focus the following discussion on blockers of L-type VGCC and SOCC. Blockers of VGCC include DHPs (e.g., NIF), phenylalkylamines (e.g., verapamil), and benzothiazepines (e.g., diltiazem). Inhibitors of SOCC include imidazoles (e.g., SKF-96365) and tricyclics (e.g., trifluoperazine). Unfortunately, these agents exhibit some nonspecificity, and their actions can be complex (Harper et al., 2003; Triggle, 2003). Although selective for VGCC at nanomolar levels, DHPs inhibit capacitive Ca^{2+} entry in the micromolar range (Harper et al., 2003). Ligand-gated ion channels are also targets of DHPs. Ca^{2+} influx involving the *N*-methyl-D-aspartate receptor was inhibited by 1 to 10 μM nitrendipine (Skeen et al., 1993), nicotinic acetylcholine receptor-induced currents were abolished by 10 μM NIF (Lopez et al., 1993), and the 5-hydroxytryptamine receptor was inhibited by 10 μM nimodipine (Hargreaves et al., 1996). These findings attest to the need to examine multiple agents and to assess the effects on each step leading to downstream events. Yet, in performing experiments to order signaling steps, it is often assumed that the agents tested (e.g., CCBs) do not alter the agonist-receptor interaction. Even when this is examined, cellular membranes are commonly used, which provides a limited assessment. These considerations have led us to focus attention on the early steps in the signaling pathway, NT binding, and NT-induced IP formation in live cells.

The current study investigates the effects of CCBs on NT binding and signal transduction in PC3 cells. Screening ion channel agents for effects on NT signaling, we unexpectedly find that VGCC and SOCC blockers dramatically enhance NT binding and cause a parallel inhibition of NT-induced IP formation. We document the specificity of these effects in regard to agent and receptor, studying eight classes of CCBs and five receptors. We find that the efficacy order to enhance NT binding is similar to that for inhibition of IP production, and these effects display similar receptor selectivity. Detailed studies examine the effects on NTR1 and investigate the involvement of Ca^{2+}-dependent PLC(s).

Materials and Methods

Materials. The radiochemicals, [^{125}I]sodium iodide (2000 Ci/mmol), [1,2-^{3}H(N)]myoinositol (60 mCi/mmol), and [^{45}Ca]calcium chloride (>10 Ci/g) were obtained from PerkinElmer Life Sciences (Boston, MA). Phloretin, 2-aminoethoxydiphenylborate (2-APB), tetrandrine, human EGF, ω-conotoxin, ionomycin, thapsigargin, and veratridine were purchased from Calbiochem (San Diego, CA). Glibenclamide, diazoxide, ryanodine, dantrolene, and 1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl]-1*H*-imidazole (SKF-96365) were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Des-Gly-[Phaa^1,D-Tyr(Et)^2,Lys^6,Arg^8]vasopressin (HOLVA) was purchased from Peninsula Laboratories (Belmont, CA). Nimodipine, verapamil, diltiazem, NT, NIF, miconazole, tetraethylammonium, flunarizine, phenylarsine oxide, amiloride, pindolol, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). [4-azido-Phe^6]NT was synthesized using reagents obtained from Novabiochem (San Diego, CA). SR48692 was generously provided by Danielle Gully at Sanofi-Synthelabo (Toulouse, France). Stocks of test agents were prepared daily (10 mM in DMSO) and diluted into Locke's solution just before use, except for SKF-96365, miconazole, and trifluoperazine (dissolved in Locke's solution).

Iodinations. Iodinations of ligands (EGF, 3 nmol; all others, 15 nmol) were performed using chloramine T (10 μg) as described (Carraway et al., 1993). All reactions were stopped using sodium metabisulfite (30 μg) except for EGF (stopped by dilution). The monoiodinated products were purified by reverse-phase HPLC using μBondapak-C18 (3.9 × 300 mm column) eluted at 1.5 ml/min with a linear gradient (60 min) from 0.1% trifluoroacetic acid to 60% CH_3CN. The specific activity of the purified ^{125}I-NT was 1000 to 2000 cpm/fmol as determined by radioimmunoassay (Carraway et al., 1993).

Binding to PC3 Cells. PC3 cells, obtained from the American Type Culture Collection (Manassas, VA) were maintained (passage 4) by our tissue culture facility at the University of Massachusetts Medical School (Seethalakshmi et al., 1997). Cells, passaged no more than 30 times, were grown to 95% confluency in 24-well culture plates. For binding studies, cells were washed with 2 ml/well Hepes-buffered Locke-BSA (Locke) (148 mM NaCl, 5.6 mM KCl, 6.3 mM Hepes, 2.4 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA; pH 7.4). Equilibrium binding at 37°C was performed for 25 min using 10^5 cpm/ml of each ^{125}I-labeled ligand in 1.0 ml Locke with varying amounts of unlabeled ligand (0–1 μM). The reaction was stopped on ice for 15 min, the medium was aspirated, and the cells were washed twice with 2 ml and once with 4 ml of ice-cold saline. During this 5-min washing procedure dissociation of ^{125}I-NT from cell receptors was <1%. Total cellular binding was assessed by measuring radioactivity and protein (Bio-Rad assay; BSA standard) in cells extracted in 0.6 ml of 0.2 M NaOH. A Packard 10-well gamma counter was used to measure radioactivity. Specific binding was defined as that displaceable by 1 μM ligand.

Cell surface binding and internalization of ^{125}I-NT were assessed by washing cells at 22°C for 2 min with 0.6 ml of 0.2 M acetic acid, 0.5 M NaCl, pH 3.0 (Beaudet et al., 1994). Binding at 4°C achieved equilibrium within 3 h, at which time >90% of the radioactivity was on the cell surface. Binding at 37°C reached equilibrium in 25 min, at which time ≅70% of total binding was internalized. To measure rates of internalization for ^{125}I-NT prebound to cells, the following procedure was used. ^{125}I-NT (10^5 cpm) was prebound to PC3 cells in

24-well plates at 4°C for 3 h. After washing the cells three times in ice-cold phosphate-buffered saline (PBS), >90% of ^{125}I-NT was located on the cell surface as determined by acid washing. Agents (10 mM in DMSO) were diluted to 50 μM in Locke and incubated with the cells at 37°C for 5 min. The control was 0.5% DMSO. Cell-surface and internalized ^{125}I-NT were measured, and the percentage of internalization per minute was calculated.

Binding displacement curves were constructed for each set of treatments and binding parameters were determined by Scatchard analysis. The K_i value was determined by using the equation $K_i = IC_{50}/1 + [L]/K_d$, where K_d and $[L]$ are the dissociation constant and the concentration of the ligand, respectively (Cheng and Prusoff, 1973).

Assessment of Binding Assay Artifacts. CCBs did not alter the ability of cells to adhere to plates, as evidenced by protein assay. Typically, each well contained 188 ± 11 μg (control, $n = 6$), 183 ± 10 μg (50 μM NIF, $n = 6$), 190 ± 12 μg (50 μM phloretin, $n = 6$), and 181 ± 11 μg of protein (100 μM verapamil, $n = 6$) after binding and washing.

CCBs did not alter the stability of ^{125}I-NT during binding at 37°C. After binding at 37°C, >90% of the radioactivity in the medium eluted during HPLC at the position of ^{125}I-NT for cells incubated in buffer, 50 μM NIF, or 100 μM verapamil. HPLC was performed at 1.5 ml/min on μBondapak-C18 (8 × 100 mm) with linear gradient (20 min) from 0.1% trifluoroacetic acid to 30% CH_3CN. ^{125}I-NT eluted at 25.0 min. During binding at 37°C, ≅4% of the added ^{125}I-NT was bound to the cells. Therefore, the medium was sampled in time and tested for the ability to bind to fresh cells. The loss of binding ability was ≅5% after 25 min. The protease inhibitors *o*-phenanthroline and PMSF (0.5 mM), had no effects on HPLC profiles and on loss of binding ability over time.

CCBs did not alter the dissociation rate of ^{125}I-NT from cellular receptors during washing with ice-cold saline. When cells were labeled with ^{125}I-NT in buffer, 50 μM NIF, or 100 μM verapamil, dissociation of cell-associated radioactivity was negligible (<6%) during incubation in ice-cold saline for 15 min.

Binding to PC3 Cell Membranes. PC3 cell membranes were prepared and collected by centrifugation at 30,000*g* as described by us (Seethalakshmi et al., 1997). Binding of ^{125}I-NT (10^5 cpm) to membranes (10–50 μg) was performed at 20°C for 60 min in 10 mM Tris-HCl (pH 7.5), containing 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% BSA, and protease inhibitors as described. Membranes were collected and washed onto glass fiber (GF-B) filters using a Brandel cell harvester, and the filters were counted (Seethalakshmi et al., 1997).

Cross-Linking of ^{125}I-[4-azido-Phe^6]-NT to NTR1. [4-azido-Phe^6]-NT was iodinated and purified by HPLC to ≅1500 Ci/mmol. PC3 cells in 10-cm dishes were incubated with 0.3×10^6 cpm/ml ^{125}I-[4-azido-Phe^6]-NT in 8 ml of Locke for 25 min at 37°C in the presence and absence of Ca^{2+} channel agents; 1 μM NT was added to controls. Cells were placed on ice for 30 min, irradiated at 254 nm with a hand-held UV light for 5 min at 3 cm, washed in ice-cold PBS, and lysed in 10 mM Hepes, 1 mM EDTA, 0.5 mM *o*-phenanthroline, PMSF, and *N*-tosyl-L-phenylalanine chloromethyl ketone (pH 7.4). Membranes, collected by centrifugation (30,000*g*, 20 min) were solubilized in 250 μl of 50 mM Tris buffer (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, and 5% glycerol at 4°C for 2 h. Solubilized NTR1, diluted 2-fold in buffer without detergent, was immunoprecipitated by addition of our rabbit antiserum (Ab-NTR1) toward the C-terminal 15 residues of human NTR1 (final 1:100). During Western blotting, Ab-NTR1 detected two major bands in extracts of PC3 cells, the parent protein of 50 kDa and a breakdown product of 33 kDa, in keeping with results in other cells (Boudin et al., 1995). After 18 h at 4°C, protein A-agarose (10 mg; Sigma-Aldrich) was added for 6 h. After the agarose beads were washed three times with PBS at 4°C, associated radioactivity was measured using a gamma counter. Usually the immunoprecipitate contained ≅5% of the solubilized cpm for samples prepared in the absence of NT. SDS-PAGE was used in some cases to validate that the radiolabeled material represented NTR1. For this, the beads were boiled for 5 min in an equal volume of 2× SDS sample buffer, and extracts were subjected to SDS-PAGE using 10% polyacrylamide gels, followed by autoradiography.

Western Blotting. PC3 cells in 60-mm dishes were washed in Locke containing inhibitors 0.5 mM EDTA, 0.5 μM PMSF, 0.5 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.5 μM *o*-phenanthroline. Cells were lysed in 100 μl of 2× SDS loading buffer with inhibitors, scraped into microfuge tubes, and sonicated (20 s) on ice. Membranes were isolated from regions of adult rat brains (Carraway et al., 1993), and P2 pellets were extracted in 2× SDS loading buffer and sonicated. Cell and tissue extracts were boiled 5 min and separated by SDS-PAGE on 10% polyacrylamide minigels. Proteins were electroeluted onto polyvinylidene difluoride (Immobilon P; Millipore Corporation, Bedford, MA). After blocking in 5% nonfat milk in TTBS (0.05% Tween 20, 20 mM Tris, 0.5 M NaCl) for 1 h and washing 3 times with TTBS, blots were incubated with the primary antiserum (1:1000) in blocking buffer for 18 h at 4°C. Our rabbit antiserum (Ab-NTR1) was raised using a synthetic peptide corresponding to residues 398–418 of human NTR1 conjugated to keyhole limpet hemocyanin. The antibodies were affinity-purified before use. Blots were washed in TTBS, then incubated with horseradish peroxidase-linked goat anti-rabbit antibody (1:1000) for 1 h at 20°C, and washed again in TTBS. Enhanced chemiluminescence was performed according to the manufacturer's instructions (Santa Cruz Biochemicals, Santa Cruz, CA). After stripping (1 h at 70°C in 62.5 mM Tris-HCl, 2% SDS, 0.1 M β-mercaptoethanol, pH 6.8) and washing in TBS, blots were reprobed with antigen-adsorbed antisera to validate the results.

Influx of $^{45}Ca^{2+}$ into PC3 Cells. The method of Katsura et al. (2000) was used to measure $^{45}Ca^{2+}$ influx in response to NT. Briefly, confluent PC3 cells in 24-well dishes were washed with Ca^{2+}-free Locke and pretreated for 10 min with 0 to 36 μM NIF (600 μl/well). The reaction was initiated by the addition of 200 μl of NT followed in 2 min by 2.5 mM $CaCl_2$ (5 μCi $^{45}Ca^{2+}$/well). After 8 min, the cells were washed three times with ice-cold Locke and solubilized in 1.0 ml of 0.25 M NaOH. The cell extract was neutralized with acetic acid, and an aliquot was subjected to liquid scintillation spectrometry to measure $^{45}Ca^{2+}$ radioactivity.

Measurement of IP Formation. IP formation was measured by the method of Chen and Chen (1999) wherein [^{3}H]inositol was used to label the phosphoinositide pool. PC3 cells in 24-well plates were incubated 48 h with myo-[^{3}H]inositol (2.5 μCi/ml) in medium 199 and 5% fetal calf serum. Medium 199 (Difco, Detroit, MI) was chosen because of its low inositol content. After washing with 2 ml of Locke, cells were preincubated 10 min with test agents in Locke and 15 mM LiCl, and reactions were started by adding a maximal dose of NT (30 nM) or vehicle. After 30 min at 37°C, the medium was aspirated, ice-cold 0.1 M formic acid in methanol (1 ml) was added, and plates were placed at −20°C overnight. Samples were transferred to tubes using 2× 2-ml water washes, and [^{3}H]IP was adsorbed to 0.25 ml AG-1 × 8 slurry (formate form; Bio-Rad, Hercules, CA), which was washed five times in 5 mM myoinositol (5 ml) and eluted in 0.75 ml of 1.5 M ammonium formate and 0.1 M formic acid. Scintillation counting was performed on 0.5 ml of eluate in 5 ml of Ecoscint (National Diagnostics, Atlanta, GA). For experiments involving removal of Ca^{2+} from the buffer, cells were washed with Ca^{2+}-free buffer and used immediately to minimize any disturbance to internal Ca^{2+} stores.

Statistics. Statistical comparisons were made using the Student's *t* test. Results were calculated as mean ± S.E.M. and $p < 0.05$ was considered significant.

Results

CCBs Enhanced Cellular Binding of NT. Specific binding of ^{125}I-NT (10^5 cpm/ml) to PC3 cells at 37°C was >95% of total binding and was 16.8 ± 0.81 cpm ^{125}I-NT bound/μg

protein (n = 12), which corresponded to ≅3000 cpm ^{125}I-NT bound per well. Table 1 gives the binding parameters determined for NT binding to PC3 cells. Data given under *Materials and Methods* attests to the validity of the assay, showing that the ^{125}I-NT remained intact during incubation and that dissociation did not occur during washing. CCBs did not alter these conditions.

CCBs (e.g., NIF, phloretin, and verapamil) increased the apparent rate[1] of and the steady-state level of NT binding to PC3 cells (Fig. 1A). NIF enhanced specific binding without altering nonspecific binding, and it was effective across a 10-fold range in cell density (Fig. 1B). Similar effects were displayed by five L-type VGCC blockers, two L-type/T-type VGCC blockers, and two blockers of SOCC, representing seven different classes of chemicals. The order of efficacy (NIF > phloretin > verapamil > diltiazem) for VGCC blockers was similar to that for peripheral vasodilation (Triggle, 1999). NT binding was increased up to 3.1-fold by NIF, 2.9-fold by phloretin, 2.0-fold by verapamil, and 1.4-fold by diltiazem (Fig. 1C). Nimodipine and NIF were the most potent agents, elevating NT binding at submicromolar concentrations [control, 100 ± 4%; 0.3 μM nimodipine, 116 ± 5% (p < 0.05); 0.9 μM NIF, 115 ± 5% (p < 0.05)]. Although less specific CCBs (flunarizine, tetrandrine, trifluoperazine, and chlorpromazine) had only modest effects (Table 2), well defined blockers of SOCC (SKF-96365, miconazole) enhanced NT-binding up to 2.9-fold (Fig. 1D; Table 2).

CCBs Inhibited NT-Induced IP Formation. NT increased IP formation ≅5-fold in PC3 cells with an EC_{50} value of ≅1 nM (Fig. 2A). L-type VGCC blockers inhibited the response to a maximal dose of NT (Fig. 2B), with an efficacy order (NIF > phloretin > verapamil) similar to that for enhancement of NT binding (Table 2). SOCC blockers also inhibited the response to NT (Fig. 2B), giving an efficacy order (SKF-96365 > miconazole > trifluoperazine) similar to that for enhancing NT binding (Table 2). For each of these agents the EC_{50} value for enhancing NT binding was similar to the IC_{50} value for inhibiting NT-induced IP formation (Table 2), and there was a strong statistical correlation (r^2 = 0.842). These results indicated that the drug effects on NT binding and NT-induced IP formation had a similar chemical sensitivity and/or that the two effects were linked, e.g., that one led to the other.

TABLE 1
Parameters determined for binding of ^{125}I-labeled ligands to PC3 cells

Ligand[a]	Specific Binding[b]	B_{max}[c]	K_i[c]
	% total	*fmol/mg*	*nM*
^{125}I-NT	95	158 ± 9	1.0 ± 0.07
^{125}I-[Nle^{14}]bombesin	95	1016 ± 64	0.6 ± 0.09
^{125}I-EGF	95	151 ± 11	0.6 ± 0.07
^{125}I-pindolol	66	86 ± 6	0.3 ± 0.05
^{125}I-HOLVA	77	156 ± 12	0.5 ± 0.07

[a] NT, [Nle^{14}]bombesin and EGF are agonists for NTR1, bombesin receptor, and EGF receptor, respectively. Pindolol and HOLVA are antagonists for β_2-adrenergic receptor and vasopressin (V1a) receptor, respectively.
[b] All ligands were HPLC-purified (specific activity >1000 Ci/mmol). Specific binding was measured to near-confluent cells (≅185 μg protein/well) using 10^5 cpm ^{125}I-ligand in 1.0 ml Locke's buffer (see under *Materials and Methods*).
[c] Scatchard analysis was performed using 12 ligand concentrations and results were from three to nine experiments.

Tyrosine Kinase Inhibitors Increased ^{125}I-EGF Binding to PC3 Cells. Tyrosine kinase inhibitors have been identified that specifically bind to the ATP binding site of EGFR and block kinase function (Arteaga et al., 1997). Since these drugs were known to greatly elevate EGF binding in some cancer cells (Lichtner et al., 2001), we tested their effects in PC3 cells. Initially, we showed that the PC3 cell surface displayed EGFR with high affinity for ^{125}I-EGF (Table 1). Testing the effects of tyrosine kinase inhibitors, we found that AG1478 and PD153035 increased EGF binding to PC3 cells by as much as 4.3-fold (Fig. 3A), whereas they had little effect on NT binding (Fig. 3B). In contrast, CCBs NIF and SKF-96365 had little effect on EGF binding (Fig. 3A,

[1] This does not necessarily indicate that the association rate constant is increased, since the apparent rate is a function of association, dissociation, internalization and other processes.

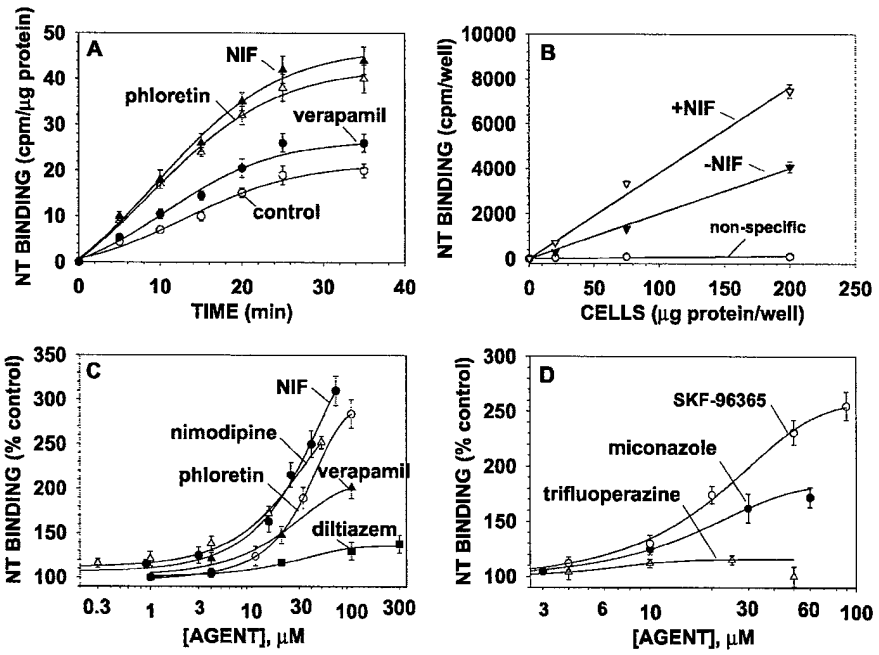

Fig. 1. CCBs increased the rate and steady-state level of NT binding to intact PC3 cells. A, PC3 cells were preincubated with 30 μM NIF, phloretin, or verapamil or with 0.3% DMSO at 37°C. After 10 min, ^{125}I-NT (10^5 cpm; 50 pM final) was added, along with NT (1 μM final) or control. Incubation continued for times indicated, reactions were stopped, and binding was expressed as cpm/μg protein. Results are for n = 3 in a typical experiment that was repeated. B, cells, diluted 1:1, 1:3, and 1:9, were plated, yielding ≅200 μg, 75 μg, and 20 μg of protein/well, respectively. Cells were preincubated 10 min with 30 μM NIF or vehicle, and NT binding (37°C, 25 min) was measured. Results are n = 6 in a typical experiment performed three times. C and D, cells were pretreated 10 min with indicated agents and NT binding was measured. The minimum dose that significantly (p < 0.05) elevated NT binding above control was 0.3 μM (nimodipine), 0.9 μM (NIF), 20 μM (verapamil), 10 μM (phloretin), 4 μM (SKF-96365), 10 μM (miconazole), and 100 μM (diltiazem). Results are from four experiments.

TABLE 2
Activity of CCBs on NT binding and NT-induced IP formation

Channel	Agent	NT Binding[a]		IP Formation[b]	
		Efficacy	EC_{50}	Efficacy	IC_{50}
		% increase	*μM*	*% decrease*	*μM*
VGCC	NIF[c]	210	15	74	15
	Phloretin[c]	186	27	70	23
	Verapamil[c]	85	43	58	53
	Diltiazem[c]	38	>300	ND	ND
	Flunarizine[d]	45	>100	ND	ND
	Tetrandrine[d]	35	>100	ND	ND
SOCC	SKF-96365	155	23	69	26
	Miconazole	75	60	54	51
	Trifluoperazine	16	>100	14	>100
	Chlorpromazine	36	>100	ND	ND

ND, no data.
[a] Efficacy was defined as the maximal percentage of increase in NT binding observed for each agent. ED_{50} was defined as the [agent] at which NT binding was increased by 80%. The data are means determined in three to eight experiments for each agent.
[b] Efficacy was defined as the maximal percentage of decrease in NT-induced IP formation observed for each agent. IC_{50} was defined as the [agent] at which IP formation was decreased by 50%. The data are means determined in three to eight experiments for each agent. nd, not determined.
[c] L-type CCBs.
[d] L-type/T-type blockers.

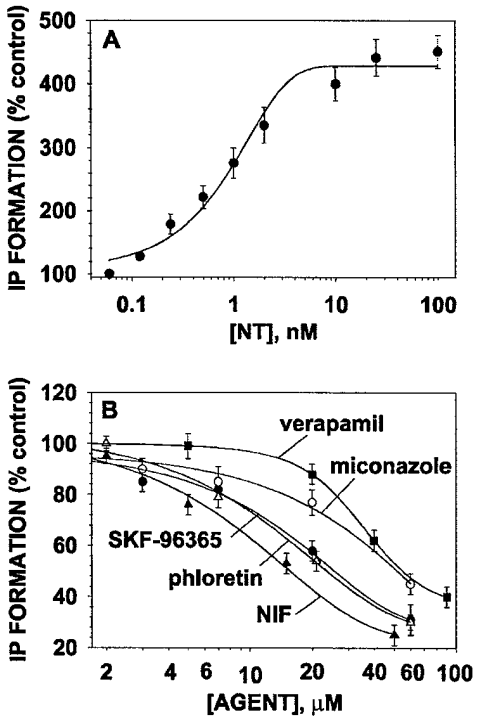

Fig. 2. NT-induced IP formation (A) was inhibited by CCBs (B). A, log dose-response plot showing that NT enhanced IP formation 4.5-fold with $EC_{50} \cong 1.0$ nM. The minimum dose of NT that significantly ($p < 0.05$) elevated IP formation above control was 0.2 nM. B, log dose-response plots showing that IP formation in response to a maximal dose of NT (30 nM) was inhibited by CCBs. The minimum dose that significantly ($p < 0.05$) decreased IP formation below control was 5 μM (NIF), 7 μM (phloretin), 7 μM (SKF-96365), 20 μM (miconazole), and 40 μM (verapamil). Results are from 10 experiments (A) and 3 to 4 experiments (B).

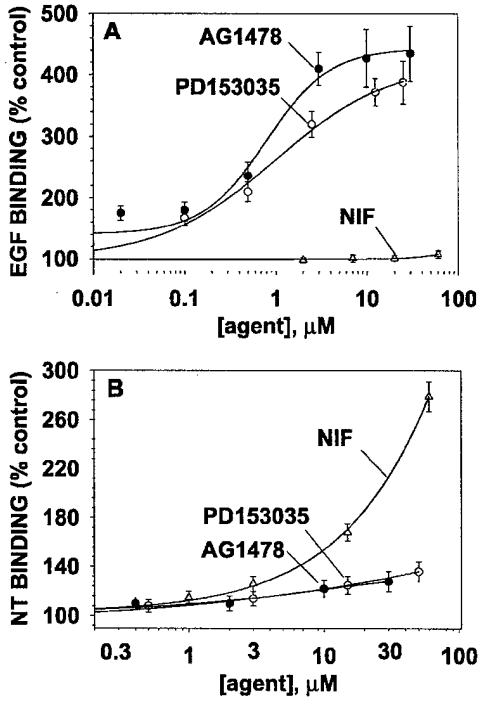

Fig. 3. Tyrosine kinase inhibitors increased ^{125}I-EGF binding to PC3 cells (A) but had little effect on NT binding (B). PC3 cells were preincubated 10 min with indicated agents, and binding was performed at 37°C. Results from five experiments (A) and three experiments (B) were expressed as percentage of control and plotted as mean ± S.E.M. A, whereas EGF binding was not altered by CCBs ($p > 0.1$), it was increased up to 4-fold by AG1478 and PD153035. B, in contrast, NT binding was increased only slightly (<35%) by tyrosine kinase inhibitors, although it was increased up to 3-fold by NIF.

Table 5), although they enhanced NT binding ≅3-fold (Figs. 1, C and D, and 3B). These results not only demonstrated specificity, but also a degree of similarity to these systems, since the elevated binding in both cases was associated with an inhibition of the response to receptor activation.

CCBs Did Not Act Directly on NTR1. Since tyrosine kinase inhibitors were thought to act directly on the EGFR to elevate binding (Lichtner et al., 2001), we wondered whether this was also the case for the effects of CCBs on NTR1. To address this, we tested the effects of CCBs on ^{125}I-NT binding to isolated PC3 cell membranes in vitro. NT binding to cell membranes was not increased by NIF, phloretin, and verapamil (Table 3), indicating that these agents were unable to act directly on NTR1. Although a key participant in the reaction might have been lost during membrane isolation, it seems more likely that there was a requirement for cellular metabolism and/or architecture. Thus, the increase in NT

TABLE 3
Effects of CCBs on NT binding to PC3 cell membranes

Agent[a]	Specific NT Binding (% Control) at Dose of Agent[b]			
	10 μM	25 μM	75 μM	100 μM
NIF	97 ± 5		102 ± 5	108 ± 8
Nimodipine	99 ± 2	101 ± 2	94 ± 4	
Phloretin	110 ± 7	105 ± 8	97 ± 8	
Verapamil	100 ± 2		101 ± 5	103 ± 4

[a] Agents were freshly dissolved in DMSO at 10 mM and diluted into binding buffer just before use.
[b] PC3 cell membranes were preincubated 10 min with agents or control, and NT binding was performed at 22°C for 60 min (see under *Materials and Methods*). Specific binding was measured in four to six experiments and expressed as percentage of control (mean ± S.E.M.). Results for the various agents were not significantly different from control ($p > 0.1$).

binding observed in live cells most likely reflected an indirect effect of CCBs, possibly by way of a change in ion movement or by some other means.

Other Channel Agents Did Not Increase NT Binding. To assess drug specificity, we tested agents toward other ion channels for effects on NT binding. We focused on agents that might alter the movement of Ca^{2+}, Na^+, and K^+ since NT binding to isolated membranes was known to be inhibited by these metal ions (Carraway et al., 1993). A variety of agents toward other types of channels did not enhance NT binding to PC3 cells (Table 4). These included an N-type Ca^{2+} channel blocker (ω-conotoxin), Ca^{2+} release inhibitors (ryanodine, dantrolene), K^+ channel blockers (glibenclamide, diazoxide, tetraethylammonium), an Na^+ channel blocker (amiloride), and an Na^+ channel opener (veratridine). These results indicated that the NT binding response displayed a degree of drug specificity.

Enhancement of Cell Binding by CCBs Was Relatively Specific to NT. To assess receptor specificity, we tested CCBs for effects on PC3 cell binding of ligands specific for other GPCRs and for EGFR. Radioreceptor assays were developed for β_2-adrenergic, bombesin, and V_{1a}-vasopressin receptors as well as for EGFR. Table 1 shows the ligands used and the binding parameters determined. For NT, bombesin, and EGF receptors agonist ligands were used; the others were antagonists. Assessing the effects of CCBs, we found that NIF, phloretin, verapamil, and SKF-96365 did not enhance β_2-adrenergic, V_{1a}-vasopressin, and EGF receptor binding to PC3 cells (Table 5). However, bombesin receptor binding was elevated slightly (≅19%) by NIF (Table 5). β_2-adrenergic receptor binding was, in fact, decreased by these

TABLE 4
Effects of various channel-directed agents on NT binding to PC3 cells

Agent[a]	Specific NT Binding (% Control) at Dose of Agent[b]			
	2 μM	10 μM	30 μM	100 μM
ω-Conotoxin	95 ± 8	104 ± 7		
Ryanodine		108 ± 7	101 ± 6	114 ± 10
Dantrolene		106 ± 6	93 ± 10	112 ± 10
Glibenclamide	102 ± 6		112 ± 9	117 ± 10
Diazoxide	106 ± 5	101 ± 5	96 ± 6	
Tetraethylammonium	103 ± 5	95 ± 6	83 ± 5	
Amiloride		108 ± 5	102 ± 6	101 ± 6
Veratridine	99 ± 2	101 ± 3	105 ± 6	

[a] Agents were freshly dissolved in DMSO at 10 mM and diluted into Locke's buffer just before use.
[b] PC3 cells were preincubated with each agent and vehicle control for 10 min; NT binding was performed at 37°C for 25 min. Specific NT binding is given as percentage of control (mean ± S.E.M.) for at least three independent experiments. Results for the various agents were not significantly different from control ($p > 0.1$).

TABLE 5
Effects of CCBs on PC3 cell binding of ligands specific for bombesin, vasopressin, β_2-adrenergic, and EGF receptors

Ligand	Agent	Specific Binding (% Control) at Dose of Agent[a]	
		12 μM	60 μM
^{125}I-[Nle^{14}]bombesin	NIF	108 ± 4	119 ± 4**
	Phloretin	104 ± 3	111 ± 4
	Verapamil	104 ± 4	104 ± 4
	SKF-96365	99 ± 2	106 ± 4
^{125}I-Pindolol[b]	NIF	105 ± 5	82 ± 3**
	Phloretin	102 ± 4	93 ± 3
	Verapamil	68 ± 5**	35 ± 6**
	SKF-96365	86 ± 2**	51 ± 2**
^{125}I-HOLVA[c]	NIF	95 ± 4	59 ± 5**
	Phloretin	92 ± 4	73 ± 4**
	Verapamil	85 ± 4*	58 ± 4**
	SKF-96365	80 ± 2**	50 ± 2**
^{125}I-EGF	NIF	100 ± 4	108 ± 4
	Phloretin	98 ± 2	95 ± 4
	Verapamil	103 ± 4	96 ± 4
	SKF-96365	103 ± 3	94 ± 3

[a] Specific binding of each ^{125}I-ligand was measured to PC3 cells (see under *Materials and Methods* and Table 1). Binding was expressed as percentage of control (mean ± S.E.M.) for three to six independent experiments.
[b] Verapamil and SKF-96365 resemble pindolol structurally. Thus, the decrease in binding was due to direct competition with the ligand (percentage of cross-reaction ≅ 0.0005). This conclusion was supported by the fact that these agents also inhibited the binding of ^{125}I-pindolol to PC3 cell membranes (see under *Materials and Methods* and Table 3). Binding (percentage of control ± S.E.M.) for three experiments in duplicate was 60 μM verapamil (9 ± 2); 60 μM SKF-96365 (18 ± 5).
[c] These agents did not resemble HOLVA structurally and they did not inhibit the binding of ^{125}I-HOLVA to PC3 cell membranes. Binding (percentage of control ± S.E.M.) for three experiments in duplicate was 60 μM verapamil (91 ± 5); 60 μM SKF-96365 (110 ± 3); 60 μM NIF (90 ± 3).
* Result was significantly different from control ($p < 0.05$).
** Result was significantly different from control ($p < 0.01$).

agents (Table 5), but this was due to a direct competition with ^{125}I-pindolol. This conclusion was based on the structural resemblance of these agents to pindolol and the fact that ^{125}I-pindolol binding to PC3 cell membranes was inhibited in a similar manner (for results, see the footnote to Table 5). Cell binding for the vasopressin receptor was also diminished by these drugs (Table 5); however, this could not be attributed to a direct competition with ^{125}I-HOLVA (Table 5 footnote). These data indicated that the robust elevation in cell binding was specific to NTR1, although the bombesin receptor responded to a lesser degree.

Inhibition of IP Formation by CCBs Was Relatively Specific to NT. To examine receptor specificity, we tested the ability of NIF to inhibit IP formation in response to GPCR agonists known to stimulate PLC. Preliminary dose-response experiments showed that a maximal dose of NT (30 nM), bombesin (20 nM), and ATP (10 μM) stimulated IP formation by ≅5-fold, ≅15-fold, and ≅17-fold, respectively. When PC3 cells were pretreated with varying amounts of NIF, we found that the response to this dose of NT was inhibited as much as ≅69%, whereas that for bombesin was inhibited ≅19%, and that for ATP was not inhibited (Fig. 4A). When the dose of each agonist was varied, we found that the percentage of inhibition by 15 μM NIF was independent of the level of stimulation. Thus, at each dose the response to NT was inhibited ≅64%, whereas that for bombesin was inhibited ≅15% and that for ATP was not inhibited (Fig. 4B). These results indicated that the robust inhibition of IP formation by

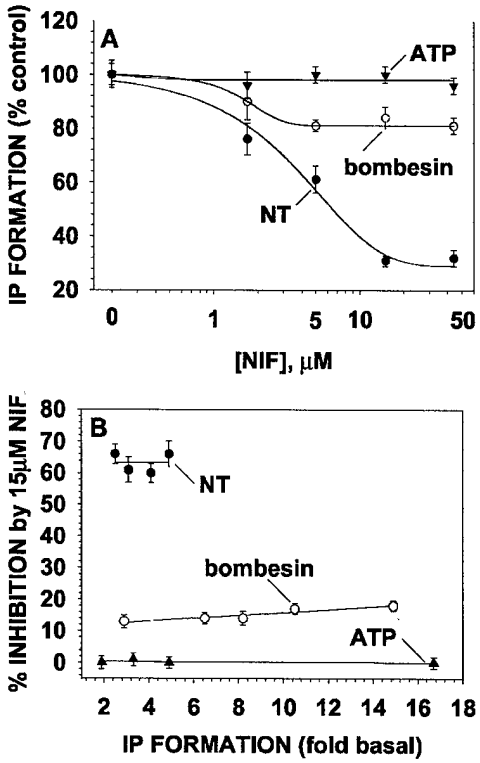

Fig. 4. NIF inhibited NT- and bombesin-, but not ATP-induced, IP formation. Agonist-induced IP formation was measured in PC3 cells. A, plots show inhibitory effects of various doses of NIF on the response to a maximal dose of NT (20 nM), bombesin (10 nM), and ATP (10 μM). Results are from three experiments. B, plots show inhibitory effects of 15 μM NIF on responses to various doses of NT (0.2–20 nM), bombesin (0.1–10 nM), and ATP (0.2–10 μM), plotted as a function of fold enhancement of basal IP formation. Results are from three experiments.

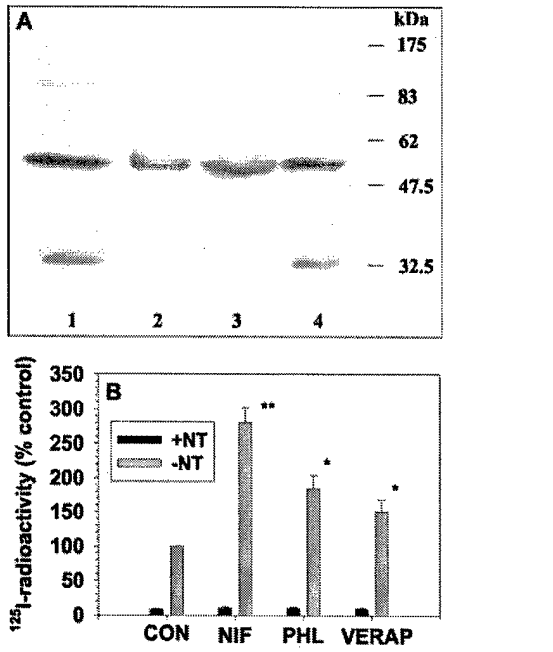

Fig. 5. CCBs enhanced photoaffinity labeling of NTR1. A, Western blot, representative of three experiments, verifying the specificity of Ab-NTR1 toward human NTR1. Lane numbering: PC3 cells (lanes 1, 4), rat cerebral cortex (lane 2), rat hypothalamus (lane 3). B, plot showing the effect of CCBs on cross-linking of ^{125}I-(4-azido-Phe^6)-NT to immunoprecipitated NTR1. PC3 cells were incubated 10 min in 50 μM NIF, 50 μM phloretin, 100 μM verapamil, or 0.5% DMSO at 37°C. ^{125}I-(4-azido-Phe^6)-NT (3×10^5 cpm/ml; 0.15 nM) was added to all dishes and 1 μM NT to some dishes. After 25 min, cells were washed, UV-irradiated, and lysed. Cell membranes were isolated and solubilized NTR1 was immunoprecipitated. After washing, precipitates were counted using a gamma counter and data were expressed relative to control (100%), which typically gave ≅5000 cpm. The drugs enhanced cross-linking 1.5- to 2.8-fold, and 1 μM NT reduced it by >90%. Results are from four experiments, except NIF (eight experiments).

NIF was specific to NT, although the response to bombesin was also inhibited to a lesser degree.

CCBs Enhanced Photoaffinity Labeling of NTR1. NTR1 is a 46-kDa protein that has been immunologically characterized (Boudin et al., 1995) and labeled using UV-activatable cross-linkers (Mazella et al., 1988). Initially, we used Western blotting to verify the specificity of our antiserum (Ab-NTR1) raised toward the C terminus of human NTR1. Whereas extracts of rat brain gave a single band at ≅50 kDa, PC3 cells gave this parent protein, along with a 33-kDa fragment (Fig. 5A), in keeping with published results (Boudin et al., 1995). Next, we used UV light to incorporate ^{125}I-(4-azido-Phe^6)-NT into PC3 cells treated with CCBs or control, and we assessed the incorporation of radioactivity into immunoprecipitated NTR1. The results (Fig. 5B) showed that the radioactivity associated with NTR1 was enhanced by NIF (2.8-fold; $p < 0.001$), phloretin (1.8-fold; $p < 0.05$), and verapamil (1.5-fold; $p < 0.05$) as compared to the control. For each agent the increase in immunoprecipitated radioactivity (Fig. 5B) was similar to the increase in NT binding to PC3 cells seen at the appropriate dose (Fig. 1C). SDS-PAGE and autoradiography on selected samples verified the presence of 50- and 33-kDa radiolabeled proteins (data not shown). These results indicated that CCBs enhanced NT binding by increasing the association of ^{125}I-NT with NTR1; however, they did not rule out possible interactions with other NT receptors.

Cell-Surface Binding versus Internalization. Cell-surface binding of ^{125}I-NT was enhanced by NIF to a similar extent when assessed by three different methods (Fig. 6). NIF increased surface binding 2.4-, 2.2-, and 2.7-fold, respectively, as measured at 4°C (Fig. 6A), 37°C in the presence of phenylarsine oxide (Fig. 6A), and 37°C by acid washing (Fig. 6B). Internalization of ^{125}I-NT was 68 to 72% of total binding in the presence or absence of NIF (Fig. 6B). In addition, the internalization rate at 37°C for cell-surface ^{125}I-NT, previously bound to cells at 4°C in the absence of drugs, was unaffected by 50 μM NIF, 50 μM phloretin, and 50 μM verapamil. Internalization rates (%/min; $n = 12$ from two experiments) were control, 8.6 ± 0.6; NIF, 8.0 ± 0.6; phloretin, 8.1 ± 0.7; verapamil, 9.2 ± 0.7, which did not differ significantly ($p > 0.1$). These results indicated that these agents increased cellular NT binding by enhancing the interaction of NT with NTR1 rather than by enhancing the internalization rate for the NT-NTR1 complex.

NTR1 Affinity versus NTR1 Number. CCBs enhanced binding and increased the steepness of the NT displacement curve. When the NT displacement data were expressed as percentage of maximal binding, CCBs shifted the displacement curves to the left by a factor of 2 to 3 (Fig. 7A). In three experiments the K_i value for NT was decreased from 0.95 ± 0.1 nM (control) to 0.36 ± 0.04 nM (50 μM NIF; $p < 0.01$), 0.40 ± 0.05 nM (50 μM phloretin; $p < 0.01$), and 0.61 ± 0.06

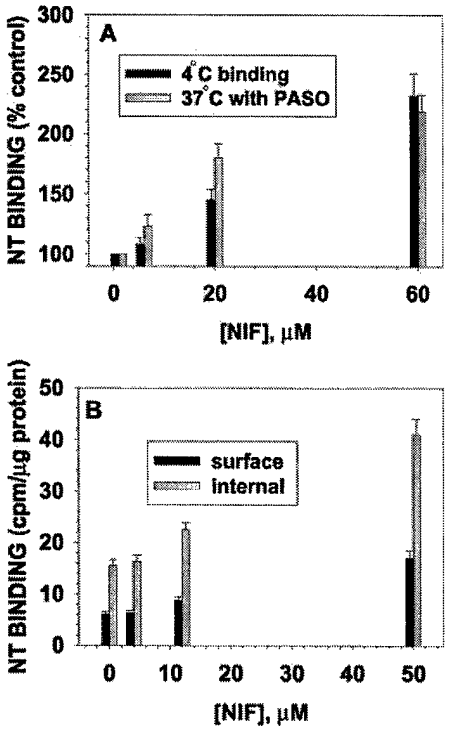

Fig. 6. NIF enhanced cell-surface binding of ^{125}I-NT (A) without altering the percentage of ^{125}I-NT internalized by PC3 cells (B). A, to study the cell-surface component of ^{125}I-NT binding we used method 1, incubation for 2 h at 4°C, and method 2, incubation at 37°C in the presence of 10 μM phenylarsine oxide. NIF enhanced cell-surface binding by 2.3-fold (method 1) and 2.2-fold (method 2). B, to study cell-surface binding and internalization we used method 3, incubation at 37°C followed by acid washing. Internalization of ^{125}I-NT was 71 ± 2% of total binding (control) and 68–72% (NIF). NIF (50 μM) enhanced cell-surface binding (2.8-fold) and internalization (2.6-fold) similarly. Results are from three experiments (A) and four experiments (B).

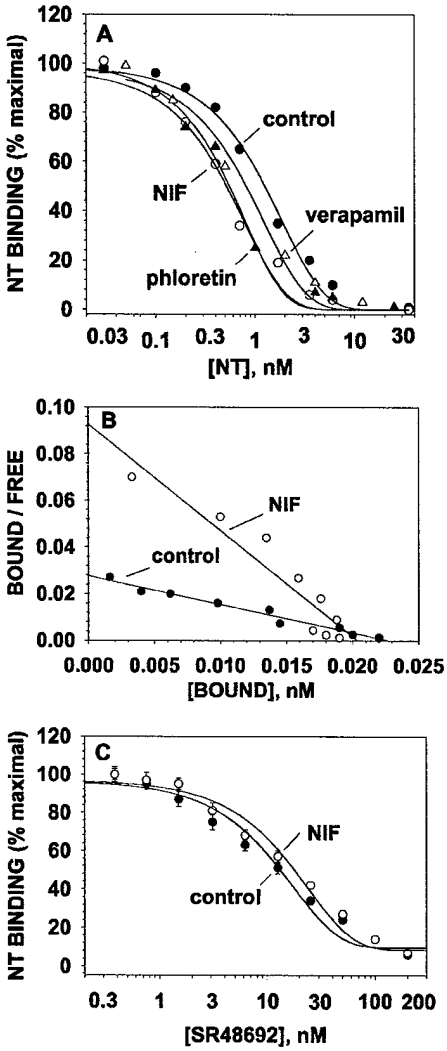

Fig. 7. Binding displacement curves (A, C) and Scatchard plots (B) for ^{125}I-NT binding to PC3 cells in the presence and absence of CCBs. Binding of ^{125}I-NT to PC3 cells (15.8 cpm/μg of protein) was increased 2.7-fold by 50 μM NIF, 2.6-fold by 50 μM phloretin, and 2.0-fold by 100 μM verapamil. A, plots show displacement of ^{125}I-NT binding by NT, in which binding was expressed as percentage of control. The agents shifted the curves to the left. The IC_{50} value for NT was 1.2 nM (control), 0.8 nM (verapamil), 0.5 nM (phloretin), and 0.5 nM (NIF). Results are from a typical experiment repeated twice. B, Scatchard plots for typical experiment showing that NIF increased NTR affinity (apparent K_i: control, 0.93 nM; NIF, 0.33 nM) without increasing receptor number (B_{max}: control, 23 fmol/well; NIF, 21 fmol/well). C, plots show displacement of ^{125}I-NT binding by SR48692. In the presence of 50 μM NIF, the curve was shifted slightly (but not significantly) to the right. Results are from typical experiment performed four times.

nM (100 μM verapamil; $p < 0.05$). Scatchard analyses indicated that NIF increased the affinity of NTR1 for NT without changing the NTR1 number (Fig. 7B). The calculated NTR1 number (158 ± 9 fmol NTR1/mg protein; $n = 9$) corresponded to ≅50,000 receptors/cell (Table 1).

In contrast, the binding displacement curve for the antagonist SR48692 was shifted slightly to the right in the presence of 50 μM NIF (Fig. 7C), although the K_i was not changed significantly (K_i: control, 12 ± 1.0 nM; NIF, 14 ± 0.8; $n = 4$; $p > 0.1$). Taken together, these results indicated that CCBs shifted NTR1 toward a state that displayed an increased affinity for the agonist NT and an unchanged affinity for the antagonist SR48692.

NIF Inhibited NT-Induced Ca^{2+} Influx. Since NT stimulated Ca^{2+} influx in Chinese hamster ovary cells transfected with NTR1 (Gailly, 1998), we tested NT for this ability in PC3 cells. NT enhanced the influx of $^{45}Ca^{2+}$ into PC3 cells, giving an EC_{50} value (≅1 nM) similar to that for NT-induced IP formation (results not shown). At doses shown to enhance NT binding (Fig. 1C) and to inhibit NT-induced IP formation (Fig. 2B), NIF inhibited the influx of $^{45}Ca^{2+}$ in response to NT (Fig. 8A).

Ca^{2+}-Dependence of NT-Induced IP Formation. Since some PLC isozymes are Ca^{2+}-dependent (Rhee and Bae, 1997), the inhibition of NT-induced IP formation by doses of NIF that diminished NT-induced Ca^{2+} influx suggested that Ca^{2+} influx might participate in the stimulation of PLC. Consistent with this, NT-induced IP formation was inhibited by omitting Ca^{2+} from Locke buffer, by adding Ca^{2+} chelator EGTA to Locke, or by adding NIF to Locke (Fig. 8B). Paradoxically, the removal of Ca^{2+} elevated basal IP production ≅2-fold (see Fig. 8 legend), perhaps by mobilizing internal Ca^{2+} stores. However, inhibition of the NT response was not due to a ceiling effect, since IP production could be elevated 15–20-fold by bombesin and ATP (see Fig. 8 legend).

Ionomycin stimulated IP formation, reproducing as much as 63% of the response to NT. IP formation (percentage of control) was 2 μM ionomycin, 139 ± 6% ($p < 0.01$); 20 μM

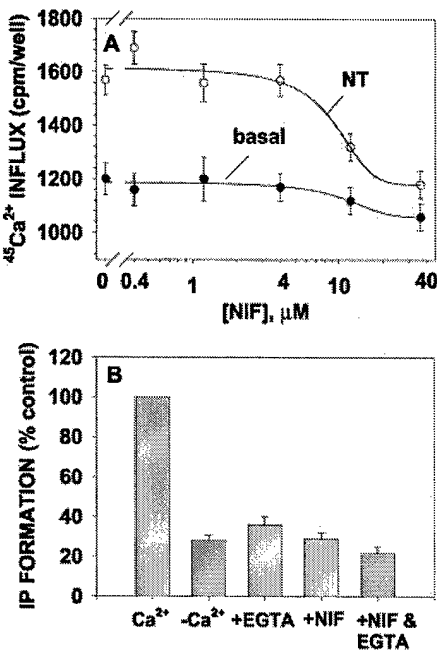

Fig. 8. NT-induced ^{45}Ca influx was inhibited by NIF (A), and NT-induced IP formation was Ca^{2+}-dependent (B). A, in experiments not shown, $^{45}Ca^{2+}$ influx into PC3 cells was enhanced ≅30% by NT (EC_{50} ≅1.2 nM). The log dose-response plot shows that a 10-min pretreatment of cells with varying doses of NIF inhibited the response to 20 nM NT (IC_{50} ≅12 μM), without much effect on basal $^{45}Ca^{2+}$ influx. Results are from three experiments. B, IP formation in PC3 cells in Locke (1 mM Ca^{2+}) was enhanced ≅4.5-fold by 20 nM NT (shown as 100% response). The response to NT was inhibited by omitting Ca^{2+} from Locke, by adding to Locke either 1.1 mM EGTA or 50 μM NIF, or 1.1 mM EGTA plus 50 μM NIF. Although basal IP formation was unaffected by NIF it was increased ≅2-fold by omitting Ca^{2+} from Locke or by adding EGTA to the Ca^{2+}-containing Locke. Inhibition of the response to NT was not due to a ceiling effect, since IP formation was stimulated ≅15-fold by 10 nM bombesin and ≅17-fold by 10 μM ATP in similar experiments. Results are from three experiments.

ionomycin, 324 ± 14% ($p < 0.01$); 30 nM NT, 457 ± 12% ($p < 0.01$); $n = 4$ experiments. When added 2 min after a maximal dose of NT (30 nM), low doses of ionomycin (2–10 μM) enhanced the response to NT. IP formation (percentage of control) was 10 μM ionomycin, 157 ± 5 ($p < 0.01$); NT, 366 ± 20 ($p < 0.01$); NT plus ionomycin, 465 ± 9 ($p < 0.001$); $n = 4$ experiments. In contrast, a maximal dose of ionomycin gave less than additive enhancement of the response to NT. IP formation (percentage of control) was 25 μM ionomycin, 322 ± 11 ($p < 0.001$); NT, 384 ± 14 ($p < 0.001$); NT plus ionomycin, 476 ± 15 ($p < 0.001$); $n = 4$ experiments. These data suggested that the inhibition of NT-induced IP formation by CCBs may have been partly attributable to a change in Ca^{2+} influx.

Ca^{2+}-Dependence of NT Binding. In six experiments, removal of Ca^{2+} from the buffer elevated NT binding significantly [NT binding (percentage of control) 2 mM EGTA, 125 ± 5; $p < 0.01$]. However, relative to the effects of CCBs (Fig. 1), this effect was very small. These data suggested that only a small part (≅10%) of the effect of Ca^{2+} channel agents on NT binding might be attributed to a change in Ca^{2+} influx.

Discussion

This is the first report that CCBs exert major effects on NTR1 binding or even, for that matter, on GPCR binding. Although NT binding to NTR1 was increased dramatically by these agents, NTR1-mediated effects on Ca^{2+} influx and IP formation were inhibited. Drugs representing three major classes of VGCC blockers enhanced NT binding, giving an efficacy order similar to that for peripheral vasodilation (Triggle, 1999). Although the most potent agents, DHPs, were regarded as specific for L-type VGCC (Triggle, 2003), their effects on NT binding and bioactivity occurred in a dose range shown to alter SOCC behavior (Harper et al., 2003). Furthermore, CCBs selective for SOCC elevated NT binding and inhibited NT-induced IP formation. Thus, the effects on NTR1 function were associated predominately with agents having the ability to block SOCC, although SOCC involvement in these actions was not proven.

Enhancement of NT binding by CCBs was drug-specific, receptor-specific, and could not be explained by enhanced tracer stability, membrane partitioning, or metabolic trapping. Under the same conditions that increased NT binding ≅3-fold and using ^{125}I-ligands with similar specific activities, binding for β_2-adrenergic, V_{1a}-vasopressin, and EGF receptors was not increased, and binding for bombesin receptor was increased <20% by CCBs. Although the results suggested that the effect was specific for NTR1, it was possible that other GPCRs could respond under proper conditions, e.g., agonist ligands might have been necessary for the enhancing effect to manifest itself. The two GPCR binding assays that gave increases in response to CCBs, NTR1 (≅200% increase) and bombesin (≅20% increase), used agonist ligands. Although difficult to understand at this time, it is interesting that the V_{1a}-vasopressin assay, which used an antagonist ligand, gave decreased binding in response to CCBs. Like the increase observed in NT binding, the decrease in vasopressin binding required intact cells (Table 5), and thus was not due to competition at the ligand binding site. Since NTR1, bombesin, and V_{1a}-vasopressin receptors signal via $G_{q/11}$, this suggests that the associated G-protein may be an important determinant of these effects. Although some CCBs decreased ^{125}I-pindolol (antagonist) binding to β_2-adrenergic receptors that signal via G_s, this was due to competition at the ligand binding site (Table 5).

That the increase in NT binding involved an enhanced interaction of NT with NTR1 was shown by photoaffinity labeling of immunoprecipitated NTR1. Augmentation of NT binding was not due to an increase in cell-surface receptors or to a change in receptor internalization. The binding of NT has been shown to initiate internalization of the NT-NTR1 complex, a process involving sortilin (Chabry et al., 1993). Stimulation of this process could conceivably lead to an apparent increase in cellular NT binding. However, we found that CCBs did not promote NT internalization and they did not change the apparent number of receptors participating in binding. Instead, the NT-displacement curve was shifted to the left, with an associated decrease in K_i and no change in NTR1 number. Classically, GPCRs display higher affinity for agonists, but not for antagonists, when they are in the coupled state as compared to the uncoupled state. CCBs increased the affinity of NTR1 for agonist NT without altering that for antagonist SR48692. Based on this, we propose that CCBs trap NTR1 in a G-protein-coupled state that exhibits increased affinity for NT. Although NT-induced IP formation was also inhibited, it is not known whether the "high-affinity" state of NTR1 exhibits a reduced ability to activate PLC.

However, Paton's rate theory of drug-receptor interaction would predict that increased affinity (associated with a decreased offset rate) would lead to decreased potency (Paton, 1961). Thus, if NTR1 is unable to release NT, it may be less efficacious.

An unexpected outcome was the finding that Ca^{2+} influx participated in the activation of PLC by NT. While other workers have shown that Ca^{2+} was required for PLC action in vitro, agonist-induced IP formation in cells was generally insensitive to removal of extracellular Ca^{2+} (Rhee and Bae, 1997). In contrast, we found 1) that NT-induced IP formation was enhanced by Ca^{2+} ionophore and inhibited by Ca^{2+} removal; 2) that Ca^{2+} ionophore stimulated IP formation, reproducing about half the NT response; and 3) that NT increased Ca^{2+} influx. Since NT can stimulate capacitative Ca^{2+} entry through SOCC (Gailly, 1998), it is likely that SOCCs contribute to the Ca^{2+} component of PLC activation by NT. Consistent with this, we found that the ability to inhibit NT-induced IP formation was associated with SOCC-directed agents. In addition, NIF inhibited NT-induced $^{45}Ca^{2+}$ uptake and NT-induced IP formation over the same dose-range. Unfortunately, we did not test other SOCC blockers for effects on $^{45}Ca^{2+}$ uptake.

Determining the PLC isotype(s) expressed by PC3 cells may be key to understanding these findings. PLCs are classified into three categories (PLCβ, PLCγ, and PLCδ) that exhibit distinct regulatory properties. While PLCβ is activated by α-subunits of $G_{q/11}$-type G-proteins and G$\beta\gamma$-subunits from other G-proteins, and PLCγ is regulated by tyrosine kinases (Rhee and Bae, 1997), PLCδ is activated by $[Ca^{2+}]$ in the physiologic range (Allen et al., 1997). Rhee and Bae (1997) proposed that PLCδ activation might occur secondary to receptor-mediated activation of PLCβ via the ensuing elevation in intracellular Ca^{2+}. This could provide an explanation for our results, given that Kim et al. (1999) have shown PLCδ1-activation mediated by the capacitative Ca^{2+} entry following bradykinin-stimulation of PC12 cells. Since PC3 cells express PLCβ and PLCδ isoforms (Carraway, unpublished results), it is possible that PLCδ might be activated by capacitative Ca^{2+} entry following NT-induced stimulation of PLCβ. Given that removing Ca^{2+} from the buffer inhibited NT-induced IP formation by ≅70%, this mechanism could account for the majority of IP formed during prolonged NT stimulation. Paradoxically, removing Ca^{2+} from the buffer was by itself a weak stimulus. Basal IP formation increased ≅2-fold when Ca^{2+} was omitted from or EGTA was added to the Ca^{2+}-containing buffer. This effect may have involved the release of Ca^{2+} from internal stores. In preliminary experiments we have shown that thapsigargin, a stimulator of internal Ca^{2+} release, elevates basal IP formation ≅2-fold.

Enhancement of NT binding by CCBs was always associated with inhibition of NT-induced IP formation. The efficacy order and potencies in these two assays were similar for the agents tested (Table 2). Furthermore, NIF altered bombesin receptor binding and bombesin-induced IP formation precisely as it did for NT, only to a lesser extent. These similar drug and receptor dependencies suggested that these effects came about coordinately or that they were separate events with similar chemical sensitivity. Supporting the latter hypothesis was the different Ca^{2+}-dependence of these effects. Whereas NT binding was largely Ca^{2+}-independent, NT-induced IP formation was partly Ca^{2+}-dependent. Although both effects were associated with SOCC-inhibiting drugs, the rank order of potency (NIF > SKF-96365 > miconazole > trifluoperazine) differed from that for inhibition of SOCC conductance (miconazole > NIF > trifluoperazine > SKF-96365) measured in HL-60 cells (Harper and Daly, 1999, 2003). One possibility is that PC3 and HL-60 cells express different Ca^{2+} channels, e.g., the six mammalian *Trp* genes can create multiple, functionally diverse Ca^{2+} channels that give complex responses to GPCR agonist activation and store depletion (Zhu et al., 1998). Another possibility is that Ca^{2+} channel occupation per se mediated CCB action, since Ca^{2+} channels interact with GPCRs (Grazzini et al., 1996) and G-proteins (De Waard et al., 1997), and since channel structure and conductance could depend on different drug properties. Another possibility is that CCBs target sites other than Ca^{2+} channels to alter NTR1 structure, and that this action alone enhances binding and obviates NT-induced IP forma-

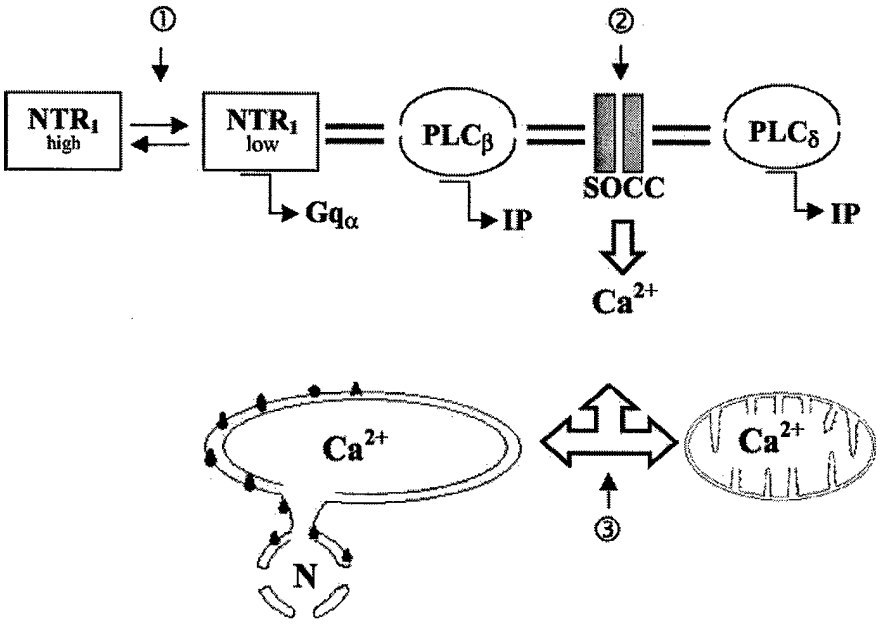

Fig. 9. Model depicting the effects of CCBs on NT binding and NT-induced IP formation. 1, by an indirect, Ca^{2+}-independent mechanism, these drugs shift NTR1 into a "high-affinity" state. If the high-affinity state of NTR1 is unable to activate PLC, this would explain the associated inhibition of NT-induced IP formation. 2, alternatively, NT-induced IP formation is inhibited by the blocking of the SOCC, which mediates the Ca^{2+} entry involved in activation of PLCδ. 3, another possibility is that these drugs alter some aspect(s) of cellular Ca^{2+} handling such that influxed Ca^{2+} is unable to activate PLCδ.

tion. At this time, the simplest explanation is that CCBs produce two effects: 1) they enhance NTR1 binding (and to a lesser extent) bombesin binding; and 2) they inhibit NT-induced (and to a lesser extent) bombesin-induced IP formation. Although changes in Ca^{2+} influx and Ca^{2+} channel interactions might contribute, especially to (2), it seems likely that other targets are also involved. These findings can be summarized as depicted in Fig. 9.

The effects of CCBs on NTR1 resemble those observed when EGFR is treated with tyrosine kinase inhibitors (Arteaga et al., 1997). Although EGF binding is increased greatly by AG1478 and PD153035 (as shown here), EGFR is unable to autophosphorylate in response to EGF and downstream responses are blocked (Lichtner et al., 2001). Tyrosine kinase inhibitors interact directly with EGFR, and the high-affinity state has been identified as an inactive dimer (Lichtner et al., 2001). CCBs do not interact directly with NTR1 since they do not increase NT binding to isolated cell membranes. However, it might be worthwhile to test the possibility that the phosphorylation state or polymerization state of NTR1 is indirectly altered by CCBs.

The DHPs, nimodipine and NIF, were the most potent (threshold dose, $\cong 1\ \mu M$) and most efficacious agents tested to elevate NT binding. NIF was also the most effective agent to inhibit NT-induced IP formation. Given that blood levels of DHPs in patients can approach the micromolar range and that DHPs concentrate in membrane fractions (Mason et al., 1992), it is possible that NT binding and bioactivity are altered in humans receiving these drugs. Whether any of the effects of these drugs on cardiovascular function involve NT is not known; however, NT is present throughout the cardiovascular system, where it can produce vasodilation and exert ionotropic and chronotropic effects (Ferris, 1989).

In conclusion, CCBs exert indirect effects in PC3 cells leading to 1) a dramatic increase in cellular NT binding and a smaller increase in bombesin binding; and 2) a dramatic inhibition of NT-induced IP formation and a smaller inhibition of the response to bombesin. Although changes in Ca^{2+} influx and Ca^{2+} channel interactions might contribute, especially to the latter response, it seems likely that other targets are involved.

Acknowledgments

We thank Li Ming Tseng, Amy Wu, and Sheryl Dooley for laboratory help, and Anne Przyborowska for graphics.

References

Allen V, Swogart P, Cheung R, Cockcroft S, and Katan M (1997) Regulation of inositol lipid-specific phospholipase Cδ by changes in Ca^{2+} ion concentrations. *Biochem J* **327:**545–552.

Arteaga CL, Ramsey TT, Shawver LK, and Guyer CA (1997) Unliganded epidermal growth factor receptor dimerization induced by direct interaction of quinazolines with the ATP binding site. *J Biol Chem* **272:**23247–23252.

Beaudet A, Mazella J, Nouel D, Chabry J, Castel MN, Laduron P, Kitabgi P, and Faure MP (1994) Internalization and intracellular mobilization of neurotensin in neuronal cells. *Biochem Pharmacol* **47:**43–52.

Belmeguenai A, Leprince J, Tonon MC, Vaudry H, and Louiset E (2002) Neurotensin modulates the amplitude and frequency of voltage-activated Ca^{2+} currents in frog pituitary melanotrophs: implication of the inositol triphosphate/protein kinase C pathway. *Eur J Neurosci* **16:**1907–1916.

Boudin H, Gruaz-Guyon A, Faure MP, Forgez P, Lhiaubet AM, Dennis M, Beaudet A, Rostene W, and Pelaprat D (1995) Immunological recognition of different forms of the neurotensin receptor in transfected cells and rat brain. *Biochem J* **305:**277–283.

Carraway RE and Mitra SP (1998) Neurotensin enhances agonist-induced cAMP accumulation in PC3 cells via Ca^{2+}-dependent adenylyl cyclase(s). *Mol Cell Endocrinol* **144:**47–57.

Carraway RE, Mitra SP, and Honeyman TW (1993) Effects of GTP analogs and metal ions on the binding of neurotensin to porcine brain membranes. *Peptides* **14:**37–45.

Chabry J, Gaudriault G, Vincent JP, and Mazella JP (1993) Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. *J Biol Chem* **268:**17138–17144.

Chen WC and Chen CC (1999) Signal transduction of arginine vasopressin-induced arachidonic acid release in H9c2 cardiac myoblasts: role of Ca^{2+} and the protein kinase C-dependent activation of p42 mitogen-activated protein kinase. *Endocrinology* **140:**1639–1648.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of enzymatic reactions. *Biochem Pharmacol* **22:**3099–3108.

De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, and Cambell KP (1997) Direct binding of G-protein βγ complex to voltage-dependent calcium channels. *Nature (Lond)* **385:**446–450.

Donoso MV, Huidobro-Toro JP, and Kullak A (1986) Involvement of calcium channels in the contractile activity of neurotensin but not acetylcholine: studies with calcium channel blockers and Bay K-8644 on the rat fundus. *Br J Pharmacol* **88:**837–846.

Ferris CF (1989) Neurotensin, in *Gastrointestinal System II* (Schultz SG and Makhlouf GM eds) pp 559–586, Oxford University Press, New York.

Gailly P (1998) Ca^{2+}-entry in CHO cells, after Ca^{2+} stores depletion, is mediated by arachidonic acid. *Cell Calcium* **24:**293–304.

Golba KS, Deja M, Imiolek P, Kotyla PJ, Biernat J, Wos S, and Herman ZS (1995) The dihydropyridines modulate neurotensin inotropic action paradoxically. *J Physiol Pharmacol* **46:**419–427.

Grazzini E, Durroux T, Payet MD, Bilodeau L, Gallo-Payet N, and Guillon G (1996) Membrane-delimited G protein-mediated coupling between V1a vasopressin receptor and dihydropyridine binding sites in rat glomerulosa cells. *Mol Pharmacol* **50:**1284–1294.

Hargreaves AC, Gunthorpe MJ, Taylor CW, and Lummis SCR (1996) Direct inhibition of 5-hydroxytryptamine$_3$ receptors by antagonists of L-type Ca^{2+} channels. *Mol Pharmacol* **50:**1284–1294.

Harper JL, Camerini-Otero CS, Li A-H, Kim S-A, Jacobson KA, and Daly JW (2003) Dihydropyridines as inhibitors of capacitive calcium entry in leukemic HL-60 cells. *Biochem Pharmacol* **65:**329–338.

Harper JL and Daly JW (1999) Inhibitors of store-operated calcium channels: imidazoles, phenothiazines and other tricyclics. *Drug Dev Res* **47:**107–117.

Hermans E and Maloteaux JM (1998) Mechanisms of regulation of neurotensin receptors. *Pharmacol Ther* **79:**89–104.

Katsura M, Higo A, Tarumi C, Tsujimura A, Takesue M, Mohri Y, Shuto K, and Ohkuma S (2000) Mechanism for increase in expression of cerebral diazepam binding inhibitor mRNA by nicotine: involvement of L-type voltage-dependent calcium channels. *Mol Brain Res* **80:**132–141.

Kim YH, Park TJ, Lee YH, Baek KJ, Suh PG, Ryu SH, and Kim KT (1999) Phospholipase C-δ 1 is activated by capacitative calcium entry that follows phospholipase C-β activation upon bradykinin stimulation. *J Biol Chem* **274:**26127–26134.

Lichtner RB, Menrad A, Sommer A, Klar U, and Schneider MR (2001) Signaling-inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinazoline tyrosine kinase inhibitors. *Cancer Res* **61:**5790–5795.

Lopez MG, Fonteriz RI, Gandia I, de la Fuente M, Villarroya M, Garcia Sancho B, and Garcia AB (1993) The nicotinic acetylcholine receptor in bovine chromaffin cell, a new target for dihydropyridines. *Eur J Pharmacol* **247:**199–207.

Mason RP, Moisey DE, and Shajenko L (1992) Cholesterol alters the binding of Ca^{2+} channel blockers to the membrane lipid bilayer. *Mol Pharmacol* **41:**315–321.

Mazella J, Chabry J, Kitabgi P, and Vincent JP (1988) Solubilization and characterization of active neurotensin receptors from mouse brain. *J Biol Chem* **263:**144–149.

Mule F and Serio R (1997) Mode and mechanism of neurotensin action in rat proximal colon. *Eur J Pharmacol* **319:**269–272.

Parekh AB (2003) Store-operated Ca^{2+} entry: dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane. *J Physiol (Lond)* **547:**333–348.

Paton WDM (1961) A theory of drug action based upon the rate of drug-receptor combination. *Proc R Soc Lond Ser B Biol Sci* **154:**21–69.

Putney JW Jr (1999) TRP, inositol 1,4,5-trisphosphate receptors and capacitive calcium entry. *Proc Natl Acad Sci USA* **96:**14669–14671.

Putney JW Jr and Bird GS (1993) The inositol phosphate-calcium signaling system in nonexcitable cells. *Endocr Rev* **14:**610–631.

Rhee SG and Bae YS (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem* **272:**15045–15048.

Rostene WH and Alexander MJ (1997) Neurotensin and neuroendocrine regulation. *Front Neuroendocrinol* **18:**115–173.

Seethalakshmi L, Mitra SP, Dobner PR, Menon M, and Carraway RE (1997) Neurotensin receptor expression in prostate cancer cell line and growth effect of NT at physiological concentrations. *Prostate* **31:**183–192.

Skeen GA, Twyman RE, and White HS (1993) The dihydropyridine nitrenipine modulates N-methyl-D-aspartate receptor channel function in mammalian neurons. *Mol Pharmacol* **44:**443–450.

Triggle DJ (1999) The pharmacology of ion channels: with particular reference to voltage-gated Ca^{2+} channels. *Eur J Pharmacol* **375:**311–325.

Triggle DJ (2003) The 1,4-dihydropyridine nucleus: a pharmacophoric template. 1. Actions at ion channels. *Mini Rev Med Chem* **3:**217–225.

Trudeau LE (2000) Neurotensin regulates intracellular calcium in ventral tegmental area astrocytes: evidence for the involvement of multiple receptors. *Neuroscience* **97:**293–302.

Vincent JP, Mazella J, and Kitabgi P (1999) Neurotensin and neurotensin receptors. *Trends Pharmacol Sci* **20:**302–309.

Zhu X, Jiang M, and Birnbaumer L (1998) Receptor-activated Ca^{2+} influx via human Trp3 stably expressed in human embryonic kidney (HEK) 293 cells. *J Biol Chem* **273:**133–142.

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0022-3565/04/3091-92–101$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 309:92–101, 2004

Vol. 309, No. 1
60442/1135044
Printed in U.S.A.

Polyphenolic Antioxidants Mimic the Effects of 1,4-Dihydropyridines on Neurotensin Receptor Function in PC3 Cells

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Received September 24, 2003; accepted December 16, 2003

ABSTRACT

This study aimed to determine the mechanism(s) by which 1,4-dihydropyridine Ca^{2+} channel blockers (DHPs) enhance the binding of neurotensin (NT) to prostate cancer PC3 cells and inhibit NT-induced inositol phosphate formation. Earlier work indicated that these effects, which involved the G protein-coupled NT receptor NTR1, were indirect and required cellular metabolism or architecture. At the micromolar concentrations used, DHPs can block voltage-sensitive and store-operated Ca^{2+} channels, K^+ channels, and Na^+ channels, and can inhibit lipid peroxidation. By varying $[Ca^{2+}]$ and testing the effects of stimulators and inhibitors of Ca^{2+} influx and internal Ca^{2+} release, we determined that although DHPs may have inhibited inositol phosphate formation partly by blocking Ca^{2+} influx, the effect on NT binding was Ca^{2+}-independent. By varying $[K^+]$ and $[Na^+]$, we showed that these ions did not contribute to either effect. For a series of DHPs, the activity order for effects on NTR1 function followed that for antioxidant ability. Antioxidant polyphenols (luteolin and resveratrol) mimicked the effects of DHPs and showed structural similarity to DHPs. Antioxidants with equal redox ability, but without structural similarity to DHPs (such as α-tocopherol, riboflavin, and *N*-acetyl-cysteine) were without effect. A flavoprotein oxidase inhibitor (diphenylene iodonium) and a hydroxy radical scavenger (butylated hydroxy anisole) also displayed the effects of DHPs. In conclusion, DHPs indirectly alter NTR1 function in live cells by a mechanism that depends on the drug's ability to donate hydrogen but does not simply involve sulfhydryl reduction.

Neurotensin (NT), a regulatory peptide found in brain and intestine (Carraway and Leeman, 1976), exerts several biological effects by way of its G protein-coupled receptor NTR1 (Vincent et al., 1999) located on neurons, endocrine cells, and smooth muscle cells (Ferris, 1989; Rostene and Alexander, 1997). Because NTR1 is often coupled to $G_{q/11}$, one of the pathways activated by NT involves phosphatidylinositol-specific phospholipase C (PLC)-mediated formation of inositol phosphates (IPs) and the release of intracellular Ca^{2+} (Hermans and Maloteaux, 1998). NT also stimulates an influx of Ca^{2+} into excitable (Trudeau, 2000) and nonexcitable cells (Gailly, 1998), and as a consequence, some of its effects are inhibited by Ca^{2+} channel blockers (CCBs). For example, the contractile effect of NT on intestinal smooth muscle (Mule and Serio, 1997) is antagonized by nifedipine, a blocker of voltage-gated Ca^{2+} channels (VGCCs). However, the effects of CCBs in these systems are not well defined, and some results are controversial. In guinea pig atria, nifedipine alters the ionotropic response to NT but investigators question whether the effect depends on Ca^{2+} influx (Golba et al., 1995). Also inconsistent is the fact that NT inhibits, rather than stimulates, VGCC currents in frog melanotrophs (Belmeguenai et al., 2002). These contradictory findings have led us to hypothesize that CCBs can alter NT signaling by exerting effects that do not involve Ca^{2+} channels.

It is well established that CCBs in the 1,4-dihydropyridine (DHP) class, such as nifedipine and nimodipine, can affect multiple targets (Triggle, 2003). Used in the submicromolar

This work was supported by Department of Defense Grant DAMD17-00-1-0528 and National Institutes of Health Center Grant DK32520, although the opinions expressed are not necessarily those of the Department of Defense and the National Institutes of Health.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

DOI: 10.1124/jpet.103.060442.

ABBREVIATIONS: NT, neurotensin; NTR1, neurotensin receptor subtype 1; PLC, phosphatidylinositol-specific phospholipase C; IP, inositol phosphate; CCB, Ca^{2+} channel blocker; VGCC, voltage-gated Ca^{2+} channel; DHP, 1,4-dihydropyridine; SOCC, store-operated Ca^{2+} channel; DTT, dithiothreitol; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester; BSA, bovine serum albumin; ROS, reactive oxygen species; DPI, diphenylene iodonium; BHA, butylated hydroxy anisole; SKF-96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole; FPL-64176, 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1*H*-pyrrole-3-carboxylic acid methyl ester; (−)-BayK-8644, *S*(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4[2-(trifluoromethyl)phenyl]-3-pyridine-carboxylic acid methyl ester.

range, DHPs are relatively specific VGCC blockers; however, in the micromolar range, they block store-operated Ca^{2+} channels (SOCCs) (Harper et al., 2003), voltage-dependent K^+ channels (Hatano et al., 2003), Na^+ channels (Yatani et al., 1988), and ligand-gated ion channels (Lopez et al., 1993). In addition, DHPs are powerful antioxidants that can inhibit lipid peroxidation (Diaz-Araya et al., 1998) and protect cells against oxidative injury (Mak et al., 2002).

In the first article of this series (Carraway et al., 2003), we reported that a variety of CCBs dose responsively enhanced the binding of ^{125}I-NT to NTR1 in prostate cancer PC3 cells, whereas they inhibited NT-induced IP formation. The effects were drug-specific, receptor-specific, and indirect, suggesting an involvement of selective cellular mediators and a requirement for cellular metabolism or architecture. Implicating Ca^{2+} channel(s) and/or Ca^{2+}-dependent step(s) was the fact that IP formation required Ca^{2+} in the medium and that NT caused an influx of Ca^{2+} into the cells. The most potent agents were VGCC blockers (nifedipine and nimodipine); however, their effects on NTR1 function occurred in a dose range shown by others to block SOCC. Because NT was shown to release Ca^{2+} from internal stores and to stimulate SOCC-mediated store refilling, this suggested that CCBs altered NT binding and NT-induced IP formation by blocking SOCC function. Although several relatively selective SOCC blockers were also able to alter NTR1 function, the rank order of potency (nifedipine > SKF-96365 > miconazole > trifluoperazine) did not agree with published results for inhibition of SOCC conductance. Furthermore, the effect of nifedipine on NT binding occurred without Ca^{2+} in the medium. We concluded that a number of possible explanations should be considered: 1) Ca^{2+} channel occupation per se, not Ca^{2+} influx, might have mediated the effects of DHPs on NTR1 function; 2) the effects of DHPs could have involved effects on Na^+ or K^+ channels, because NT binding is modulated by these ions (Carraway et al., 1993); and 3) the antioxidative property of DHPs might have been the basis for these actions, because NT binding is sulfhydryl-dependent (Mitra and Carraway, 1993).

To clarify this issue, we have now performed studies to elucidate the mechanism(s) by which DHPs alter NTR1 function in PC3 cells. The role of Ca^{2+} and Ca^{2+} channels was examined by testing the effects of agents that altered cellular [Ca^{2+}] and/or perturbed Ca^{2+} channel structure. We tested the possibility that Na^+ and/or K^+ channels participated by varying the concentrations of these ions. The importance of redox activity was assessed by comparing the effects of a series of DHPs with known antioxidative ability and by testing other antioxidants. Our results support the hypothesis that DHPs alter NTR1 function via an indirect, redox-sensitive mechanism that does not seem to involve reduction of sulfhydryl groups.

Materials and Methods

Materials. Radiochemicals, ^{125}I-sodium iodide (2000 Ci/mmol) and [1,2-^{3}H(N)]-myo-inositol (60 mCi/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). Ionomycin, thapsigargin, UTP, phorbol 12-myristate 13-acetate, resveratrol, and luteolin were from Calbiochem (San Diego, CA). FPL-64176 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). NT, nifedipine, nimodipine, (−)-BayK-8644, felodipine, nicardipine, BHA, DTT, EGTA, BAPTA-AM, α-tocopherol, β-carotene, ascorbic acid, riboflavin, thiamine, pyridoxine, menadione, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Compound 1, 1-ethyl-1,4-dihydro-2,6-dimethyl-4-(4-methoxyphenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, was a generous gift from Dr. Juan Arturo Squella (University of Chile, Santiago 1, Chile).

Binding to PC3 Cells. PC3 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained by our tissue culture facility (Seethalakshmi et al., 1997). Cells were grown to 95% confluence in 24-well culture plates. High-performance liquid chromatography-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us (Carraway et al., 2003). In brief, cells were washed with 2 ml/well of Hepes-buffered Locke-BSA (Locke): 148 mM NaCl, 5.6 mM KCl, 6.3 mM Hepes, 2.4 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA, pH 7.4. Stock solutions of each agent in Locke or in dimethyl sulfoxide (10 mM) were prepared just before use and were diluted to give ≤1% dimethyl sulfoxide final. Equilibrium binding at 37°C was performed for 25 min using 10^5 cpm/ml ^{125}I-NT in 1.0 ml of Locke with varying amounts of NT. The reaction was stopped on ice for 15 min, the medium was aspirated, and the cells were washed twice with 2 ml and once with 4 ml of ice-cold saline. Total cellular binding was assessed by measuring radioactivity (Packard 10-well gamma-counter) and protein (Bio-Rad assay; BSA standard; Bio-Rad, Hercules, CA) in cells extracted in 0.6 ml of 0.2 M NaOH. Specific binding, defined as that displaceable by 1 μM NT, was 95%. Binding displacement curves were constructed for each set of treatments and binding parameters were determined by Scatchard analysis and by using the Cheng-Prusoff equation $K_i = IC_{50}/1 + [L]/K_d$, where K_d and [L] are the dissociation constant and the concentration of the ligand, respectively. The sucrose buffer used in some experiments was identical to Locke except that 296 mM sucrose was substituted for the NaCl and $NaHCO_3$. The K^+ depolarization buffer used in some experiments was identical to Locke except that 60 mM KCl was substituted for 60 mM NaCl.

Binding to PC3 Cell Membranes. PC3 cell membranes were prepared and collected by centrifugation at 30,000*g* as described by us (Seethalakshmi et al., 1997). Binding of ^{125}I-NT (10^5 cpm) to membranes (10–50 μg) was performed at 20°C for 60 min in 10 mM Tris-HCl (pH 7.5), containing 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% BSA, and protease inhibitors as described previously. Membranes were collected and washed onto glass fiber (GF-B) filters using a cell harvester (Brandel Inc., Gaithersburg, MD), and the filters were counted (Carraway et al., 1993).

Measurement of IP Formation. Formation of [^{3}H]IP in response to NT was measured as described previously (Carraway et al., 2003). Briefly, PC3 cells in 24-well plates were incubated 48 h with myo-[^{3}H]inositol (2.5 μCi/ml) in medium 199, 5% fetal calf serum. After washing in Locke, cells were preincubated 10 min with varying concentrations of test agent in Locke, 15 mM LiCl. After aspiration, fresh Locke with test agent was added, and reactions were started by adding NT or control. After 30 min at 37°C, medium was aspirated, ice-cold 0.1 M formic acid in methanol (1 ml) was added and plates were placed at –20°C overnight. Samples were adsorbed to AG-1 × 8 (formate form; Bio-Rad), which was washed five times in 5 mM myo-inositol and eluted in 1.5 M ammonium formate, 0.1 M formic acid. Scintillation counting was performed in Ecoscint (National Diagnostics, Manville, NJ).

Statistics. Statistical comparisons were made using the Student's *t* test. Data were calculated as mean ± S.E.M., and $p < 0.05$ was considered significant.

Results

Dependence on Extracellular [Ca^{2+}]. Previously (Carraway et al., 2003), we reported that CCBs, particularly those in the DHP class, dose-responsively enhanced the binding of ^{125}I-NT to PC3 cells (as much as 3-fold) and inhibited

NT-induced IP formation (as much as 70%). The fact that NT caused an influx of $^{45}Ca^{2+}$ in PC3 cells that was inhibited by nifedipine suggested that DHPs might alter NTR1 function by blocking Ca^{2+} movement. To test this hypothesis, we examined the effects of Ca^{2+} chelators and Ca^{2+} ionophores on NT binding to PC3 cells, and on the ability of nifedipine to enhance NT binding. Blocking Ca^{2+} influx with 2 mM EGTA enhanced NT binding (38 ± 6% increase; $p < 0.05$), but the effect was small relative to the 200% increase by nifedipine. In addition, the ability of nifedipine to enhance NT binding persisted in the absence of extracellular Ca^{2+}, was not reversed by 20 μM ionomycin, and was not altered by chelation of intracellular Ca^{2+} using 50 μM BAPTA-AM (Fig. 1).

These results indicated that the enhancement of NT binding by DHPs was not due to a change in Ca^{2+} influx. In contrast, the inhibition of NT-induced IP formation by DHPs might have involved an effect on Ca^{2+}, because our earlier work showed that this response was Ca^{2+}-dependent (Carraway et al., 2003).

Ca^{2+} Channel Agonists versus Antagonists. The VGCC agonist (−)-BayK-8644 and the antagonist nifedipine enhanced NT binding to a similar extent (Fig. 2A). Both compounds enhanced binding by increasing NTR1 affinity, not by altering receptor number (Fig. 2B; Table 1). Another VGCC agonist, FPL-64176, known to act at a unique non-DHP site (Zheng et al., 1991), was also active, although less potent (Fig. 2A). In addition, the agonists (−)-BayK-8644 and FPL-64176 shared with the antagonist nifedipine an ability to inhibit NT-induced IP formation (Fig. 2C). For each agent, the NT dose-response relationship was shifted downward, indicating that the efficacy of NT was decreased, not its potency (Fig. 2D).

These results were consistent with the possible involvement of SOCC, but not VGCC, in the effects of DHPs on NTR1 function. Because IP formation was Ca^{2+}-dependent, the fact that these agents decreased the efficacy of NT was in keeping with their known ability to diminish SOCC conductance.

Agents in Combination Gave Additive Effects. We wondered how combinations of CCBs would interact with NTR1, especially in regard to agonist/antagonist combinations and mixtures of CCBs that bind at different sites on Ca^{2+} channels. To test this, dose-response studies were performed using combinations of antagonist nimodipine and agonist FPL-64176, which are known to bind at discrete sites. Whereas NT binding was enhanced in an additive manner at low concentrations of each drug, the results were less than additive at high concentrations (Fig. 3A). NT-induced IP formation was inhibited in an analogous manner and at high concentrations, it reached a limit at ~70% inhibition (Fig. 3B). Similar studies were performed using various combinations of nifedipine, verapamil, and diltiazem (all antagonists). Again, when low doses of these drugs were combined, additive effects were observed for the enhancement of NT binding and for the inhibition of NT-induced IP formation, whereas at high doses the effects were less than additive (data not shown). No potentiative or antagonistic effects were observed. Together, these results indicated that the drugs tested, whether Ca^{2+} channel agonists or antagonists, seemed to act in a similar manner to alter NTR1 function.

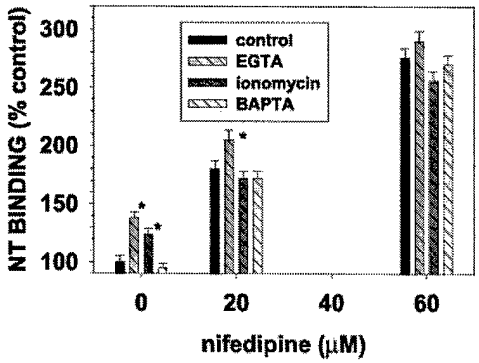


Fig. 1. Ca^{2+} chelators (EGTA and BAPTA-AM) and Ca^{2+} ionophore (ionomycin) did not prevent the enhancing effect of nifedipine on NT binding. PC3 cells were pretreated 10 min with indicated agents or with vehicle control, and NT binding was measured. Plot shows that 2 mM EGTA and 20 μM ionomycin increased NT binding by 25 to 40% but did not prevent the response to nifedipine (NIF). BAPTA-AM (50 μM) did not alter NT binding or the response to nifedipine. Results were from three experiments. Asterisk (*) indicates result was significantly different ($p < 0.05$) from appropriate control.

Effects of Ca^{2+} Channel Pertubation. Because evidence for direct "conformational coupling" of Ca^{2+} channels with some receptors existed (Grazzini et al., 1996), it was conceivable that Ca^{2+} channels might interact directly with NTR1. To examine this hypothesis, we tested treatments expected to perturb Ca^{2+} channel structure for effects on NTR1 function. VGCC can respond to membrane depolarization and Ca^{2+} feedback (Catterall, 2000). Agents that elevated cellular [Ca^{2+}], either via release of internal Ca^{2+} stores (thapsigargin and UTP) or by enhancing Ca^{2+} influx (ionomycin), increased NT binding by ~30% (Fig. 4A). BAPTA-AM, which would decrease cellular [Ca^{2+}], had little effect. Because thapsigargin, ionomycin, and UTP did not change NT binding to PC3 cell membranes (data not shown), their effects in intact cells were indirect. Cell membrane K^+-depolarization also increased NT binding (increment, ~15%), and this was associated with an inhibition of NT-induced IP formation (Fig. 4B). The dose-response relationship was shifted downward by K^+ depolarization, indicating that the efficacy of NT was decreased by ~35% (Fig. 4B).

The inhibition of NT-induced IP formation by treatments expected to perturb Ca^{2+} channels implicated VGCC and/or SOCC in this response and was consistent with the Ca^{2+} dependence of PLC (Carraway et al., 2003). However, these effects were relatively small (~1/3 that of DHP), as were the effects of these treatments on NT binding (~1/5 that of DHP). Thus, we were drawn to the idea that the effects of DHPs were, to a large degree (as much as 80%), attributable to some other property that was not necessarily related to the ability to alter Ca^{2+} channel behavior.

DHP Effects Were Not Na^+-Dependent. Because DHPs can inhibit Na^+ channels and because NT binding to PC3 cell membranes is decreased by Na^+ (Seethalakshmi et al., 1997), we considered the hypothesis that DHPs enhanced cellular binding of NT by interfering with the inhibitory effect of Na^+. Substituting sucrose for NaCl in the Locke buffer increased NT binding and shifted the NT displacement curve to the left; however, nifedipine caused a further shift to the left, even in the absence of Na^+ (Fig. 5A). Scatchard analyses showed that each of these effects was due to an increase in NTR1 affinity, without a change in NTR1 number (Table 2). Careful comparison showed that the effect of 50 μM

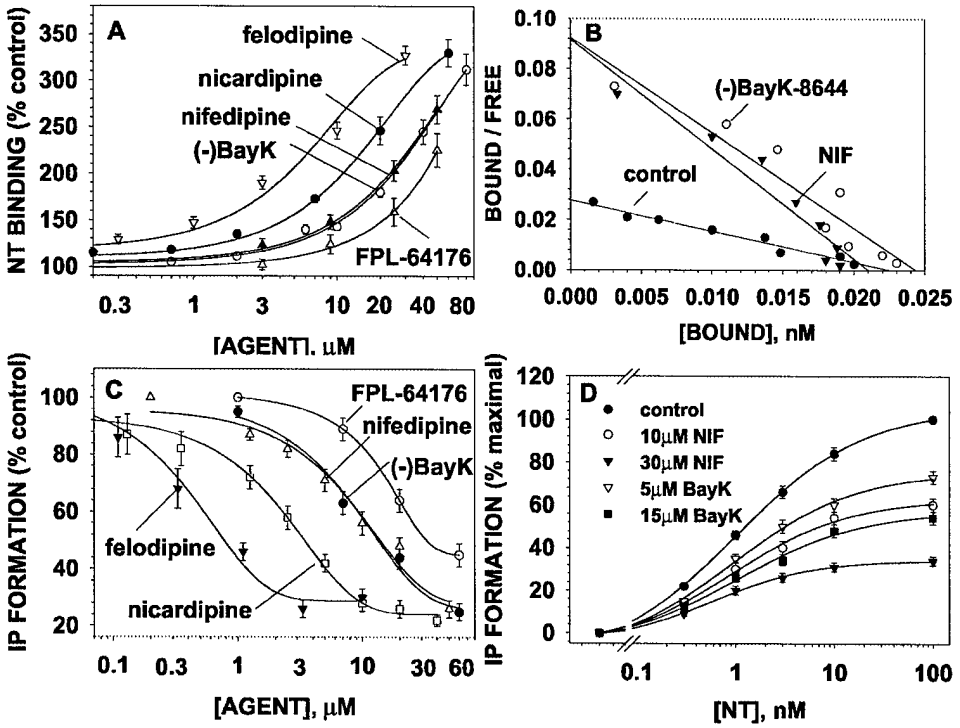


Fig. 2. Ca^{2+} channel agonists, like antagonists, enhanced NT binding (A) by increasing NTR affinity, not NTR number (B). Ca^{2+} channel agonists and antagonists inhibited NT-induced IP formation (C) by decreasing NT efficacy (D). A, cells were pretreated 10 min with indicated agents, and NT binding was measured. The minimum dose that significantly ($p < 0.05$) elevated NT binding was 0.3 μM (felodipine), 0.7 μM (nicardipine), 2 μM (nifedipine), 3 μM [(−)-BayK-8644] and 9 μM (FPL-64176). B, Scatchard plots show that NTR affinity was increased by 50 μM nifedipine and by 50 μM (−)-BayK-8644. The K_i value for NT was (nanomolar) control, 0.84; nifedipine, 0.29; and (−)-BayK-8644, 0.24. The agents did not alter NTR number (femtomoles per milligram): control, 147; nifedipine, 140; and (−)-BayK-8644, 160. C, PC3 cells were pretreated 10 min with indicated agents, and IP formation in response to 30 nM NT was measured. For control, NT increased IP formation ~5-fold. Each agent inhibited the response by 50 to 75%. The minimum dose that significantly ($p < 0.05$) decreased IP formation was 0.3 μM (felodipine), 1 μM (nicardipine), 5 μM (nifedipine), 7 μM [(−)-BayK-8644], and 20 μM (FPL-64176). D, PC3 cells were pretreated 10 min with agents indicated, and the dose response for NT-induced IP formation was measured. Note that nifedipine and (−)-BayK-8644 decreased efficacy, not potency. At [NT] ≥0.3 nM, IP formation for each treatment was significantly different from the control. In A, C, and D, results were from at least three experiments for each plot. B, typical result for an experiment that was repeated twice.

TABLE 1
Effects of CCBs and antioxidants on NT binding parameters in PC3 cells

Agent[a]	Classification	B_{max}[b]	K_i[b]
		fmol/mg	*nM*
None	Control	155 ± 11	1.0 ± 0.07
50 μM Nifedipine	VGCC antagonist	152 ± 10	0.51 ± 0.05[c]
50 μM (−)-BayK-8644	VGCC agonist	162 ± 12	0.56 ± 0.06[c]
60 μM Luteolin	Flavonoid antioxidant	164 ± 10	0.62 ± 0.05[c]
150 μM Resveratrol	Polyphenol antioxidant	171 ± 11	0.36 ± 0.04[c]

[a] PC3 cells were pretreated 10 min with indicated concentrations of each agent or vehicle control. ^{125}I-NT (10^5 cpm, 50 pM) was added and specific binding was measured at 37°C.
[b] Scatchard analyses were performed using 12 concentrations of NT. The results for B_{max} and K_i (mean ± S.E.M.) were from at least three experiments per agent.
[c] Indicates significant difference ($p < 0.05$) compared with control.

nifedipine on NT binding (~2-fold increase) and on the K_i for displacement of NT binding (~2-fold decrease) was unaffected by removal of Na^+ (Table 2). Similarly and in accordance with the binding data, NT was ~2 fold more potent in stimulating IP formation in the absence of Na^+ than in its presence (EC_{50}: Locke, 1.1 ± 0.1 nM; sucrose 0.5 ± 0.1 nM; three experiments; $p < 0.05$). However, the ability of nifedipine to inhibit NT-induced IP formation was independent of Na^+ (Fig. 5B). These results indicated that nifedipine altered NTR1 function by mechanism(s) that did not require Na^+ in the buffer.

DHP Effects Were Not K^+-Dependent. Because DHPs can inhibit K^+ channels and because K^+ inhibits NT binding to cell membranes, we performed experiments similar to those described above to assess the K^+ dependence of the effects on NTR1 function. Substitution of 60 mM KCl for NaCl in the Locke buffer did not alter the ability of nifedipine to enhance NT binding (EC_{50}: control, 22 ± 3 μM; 60 mM K^+, 24 ± 3 μM; three experiments) and to inhibit NT-induced IP formation (IC_{50}: control, 15 ± 2 μM; 60 mM K^+, 14 ± 2 μM; three experiments). Thus, these effects of nifedipine were not K^+-dependent.

Relationship to Antioxidant Activity. Because DHPs exhibit antioxidant ability (Mak et al., 2002), we wondered how this related to the effects on NTR1 function. DHPs inhibited Fe^{3+}/ascorbate-stimulated lipid peroxidation in rat

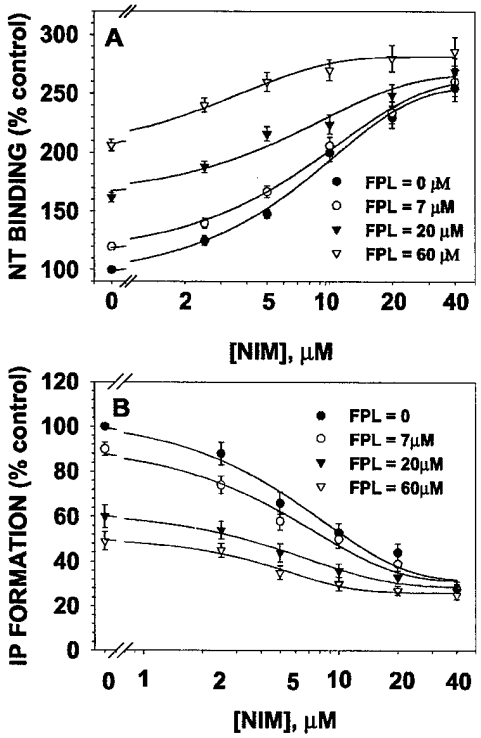


Fig. 3. Nonadditivity for effects of nimodipine (NIM) and FPL-64176 on NT binding (A) and NT-induced IP formation (B). In A, PC3 cells were pretreated 10 min with combinations of indicated agents, and NT binding was measured. Plots show that low doses of agents in combination gave additive increases in NT binding, whereas high doses gave nonadditive responses. In B, PC3 cells were pretreated 10 min with combinations of indicated agents, and IP formation was measured in response to 30 nM NT. Plots show that low doses of agents in combination gave additive decreases in IP formation, whereas high doses gave nonadditive effects. Results in A and B were from at least three experiments for each plot.

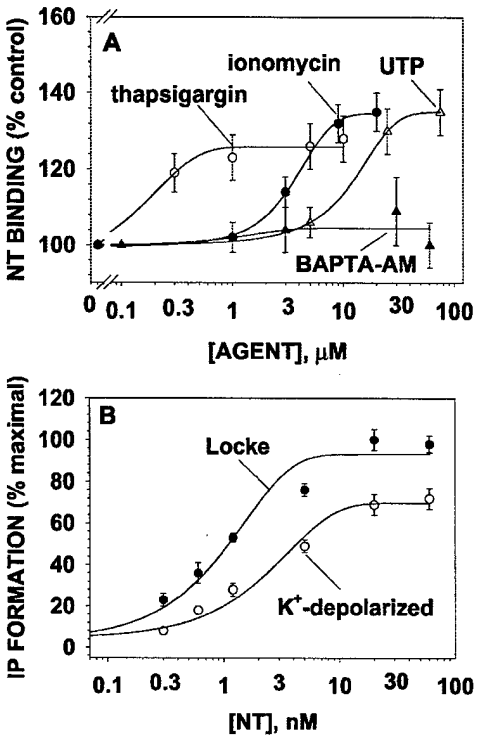


Fig. 4. Thapsigargin, ionomycin, and UTP enhanced NT binding to PC3 cells (A). Cell membrane depolarization inhibited NT-induced IP formation (B). A, PC3 cells were pretreated 10 min with agents indicated and NT binding was measured. NT binding was significantly ($p < 0.05$) elevated by thapsigargin (>0.3 μM), ionomycin (>3 μM), and UTP (>30 μM). BAPTA-AM had no effect. B, PC3 cells, labeled with [^{3}H]inositol, were placed in Locke or isotonic K^+ depolarization buffer (60 mM K^+). IP formation was measured in response to indicated amounts of NT. At each [NT], IP formation for the K^+-depolarized set was significantly different from control ($p < 0.05$). In A and B, results are from three experiments for each plot.

brain slices with activity order nicardipine > nimodipine > nifedipine (Diaz-Araya et al., 1998). The same activity order was found when these agents were compared for ability to enhance NT binding (Fig. 2A) and to inhibit NT-induced IP formation (Fig. 2C). In both systems, nicardipine was 2- to 4-fold more potent than nifedipine (Table 3). We also tested felodipine, which was 2- to 4-fold more active than nicardipine (Table 3). Although felodipine was reported to be inactive in the rat brain assay mentioned above, it was more active than nicardipine in a similar assay using myocardial membranes (Janero and Burghardt, 1989). In addition, the relative chemical reactivity of DHPs with superoxide anion was reported to be felodipine > nimodipine > nifedipine > compound 1 (Ortiz et al., 2003). For these substances, the potency to alter NT binding correlated to antioxidant activity, giving $r^2 = 0.89$ (Table 3). Compound 1, a DHP analog with *N*-ethyl in place of the NH moiety, was reported by Ortiz et al. (2003) to have a greatly reduced reactivity with superoxide (<10% that of felodipine). Here, we found that it displayed ~4% the activity of felodipine and 20 to 50% the activity of nifedipine in altering NTR function (Table 3). These results suggested that DHPs might act by some reaction(s) involving hydrogen donation.

Effects of Various Antioxidants. ROS scavengers include vitamin-like antioxidants, flavonoids, and polyphenols (Rice-Evans et al., 1996). Testing vitamin-like antioxidants on NT binding in PC3 cells, we found β-carotene, thiamine, riboflavin, pyridoxine, ascorbic acid, α-tocoferol, and tetrahydro-biopterin to be ineffective (used at 20–180 μM; $n = 3$), whereas vitamin K (menadione) had a small effect at 180 μM (percentage of control: 168 ± 8; $n = 3$; $p < 0.05$). Other antioxidants without effect included *N*-acetyl cysteine, glutathione, and sodium borohydride (used at 1–3 mM; $n = 3$); trolox, ellagic acid, (+)-catechin, (−)-epigallocatechin gallate, and rutin (used at 10–100 μM; $n = 3$).

In striking contrast were the results for the polyphenolic antioxidants luteolin (a flavonoid) and resveratrol, which displayed effects that were indistinguishable from those of DHPs. Luteolin and resveratrol enhanced NT binding (Fig. 6A), and the effect involved an increase in NTR1 affinity without a significant change in NTR1 number (Fig. 6B; Table 1). These antioxidants also inhibited NT-induced IP formation (Fig. 6C), and the effect involved a dose-dependent decrease in NT efficacy (Fig. 6D). When tested together for effects on NT binding, the response to luteolin plus nimodipine and resveratrol plus nimodipine was additive at low doses of each agent, whereas they were less than additive at high doses (data not shown). Thus, polyphenolic antioxidants mimicked the effects of DHPs and seemed to act via the same pathway.

Involvement of Sulfhydryl Groups. Because some antioxidants act by reducing sulfhydryl groups on proteins and because NT binding requires sulfhydryl groups associated

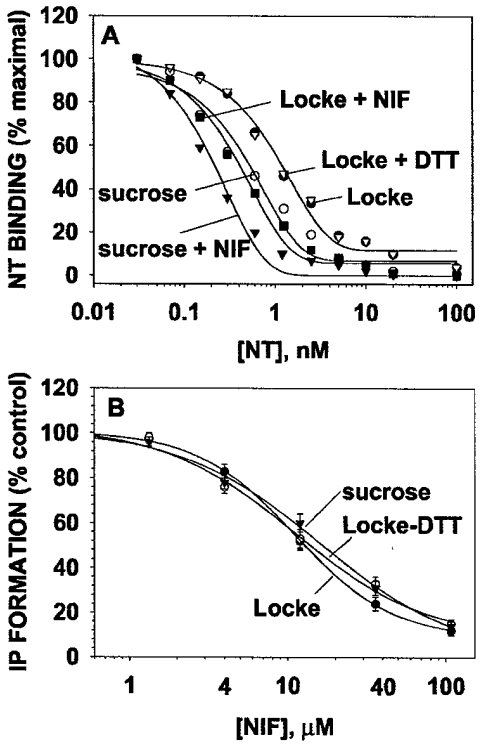


Fig. 5. Nifedipine shifted the NT binding displacement curve to the left (A) and inhibited NT-induced IP formation (B) in the presence (Locke) or absence (sucrose) of Na^+. The effects of substituting sucrose for NaCl in the buffer were determined for NT binding and NT-induced IP formation. A, maximal NT binding was higher in Locke plus nifedipine (2.2-fold), sucrose (1.7-fold), and in sucrose plus 50 μM nifedipine (3.2-fold) than in Locke or Locke plus 2 mM DTT. Plots show displacement of ^{125}I-NT binding by NT, in which NT binding was expressed as percentage of maximal. Note that the curves were shifted to the left by nifedipine and by sucrose. The IC_{50} value for NT was 1.1 nM (Locke and Locke plus DTT), 0.4 nM (nifedipine), 0.5 nM (sucrose), and 0.2 nM (sucrose plus nifedipine). Results are from a typical experiment that was repeated twice. B, PC3 cells were pretreated 10 min in Locke, Locke plus 2 mM DTT or sucrose containing the indicated amounts of nifedipine. IP formation in response to 30 nM NT was measured. Plots show that nifedipine inhibited NT-induced IP formation with a similar IC_{50} (13–16 μM) for each condition. Results are from three experiments.

with NTR1 (Mitra and Carraway, 1993), we tested the hypothesis that DHPs increase NT binding by maintaining sulfhydryl group(s) in a reduced state. Confirming the importance of sulfhydryl groups, we showed that sulfhydryl chelators, Ni^{3+} (IC_{50} of ~50 μM) and Cd^{2+} (IC_{50} of ~600 μM), inhibited NT binding to PC3 cells, and that their effects were inhibited by 2 mM DTT (data not shown).

However, in the basal state, the sulfhydryl group(s) required for NT binding to PC3 cells was primarily reduced, because NT binding was increased only slightly by 1 mM ascorbic acid (114 ± 7%; n = 4), 2 mM DTT (125 ± 8%; n = 4), and 5 mM *N*-acetyl-cysteine (107 ± 6%; n = 4). In addition, 2 mM DTT did not alter the NT displacement curve (Fig. 5A) and did not inhibit the effects of nifedipine. NT binding was enhanced similarly by 50 μM nifedipine in the presence and absence of 2 mM DTT (control, 246 ± 10%; DTT, 231 ± 11%; n = 4). NT-induced IP-formation was inhibited similarly by nifedipine in the presence and absence of DTT (Fig. 5B). Thus, DHPs acted by an antioxidant mechanism that did not involve the reduction of sulfhydryl groups in NTR1.

Involvement of Flavoprotein Dehydrogenase(s). Hypothesizing that DHPs might act by scavenging ROS produced by cellular flavoprotein dehydrogenases, we tested the effects of diphenylene iodonium (DPI), an inhibitor of these enzymes. DPI mimicked the effects of DHPs on NT binding (Fig. 6A) and NT-induced IP formation (Fig. 6C). The hydroxy-radical scavenger butylated hydroxy anisole (BHA) was also effective (Table 3). These results suggest that flavoprotein dehydrogenases and/or ROS produced by these enzymes participate in the effects of DHPs on NTR1 function.

Comparisons of Chemical Structures. The chemical structures of the DHPs and polyphenols were similar, each possessing aromatic ring structures with redox capability (Fig. 7). The order of potency (Table 3) for ability to alter NTR1 function (felodipine > nitrendipine ≅ nicardipine > nimodipine > nifedipine > luteolin > resveratrol) seemed to relate to donor group acidity (NH > OH) and to the number of conjugated double bonds. For DHPs, chloro substituents in the adjacent phenyl ring gave the highest activity (felodipine), whereas nitro in the meta position was less effective (nitrendipine, nicardipine, and nimodipine) and nitro in the ortho position was least effective (nifedipine). Luteolin and resveratrol contained conjugated π-bonded rings, which could potentially support the stability of radicals and cations (Solomons, 1994). By donating hydrogen(s), DHPs could conceivably form pyridinium or pyridine analogs with an even greater number of conjugated double bonds and potential to support radical and cation formation. The very high membrane partition coefficients displayed by DHPs (Mason et al., 1999) could determine their ability to accumulate at target site(s).

Discussion

This study investigated the mechanism(s) by which DHPs enhance NT binding and inhibit NT signaling in PC3 cells. We explored various hypotheses, and our results indicated that the effects of DHPs on NTR1 function correlated to their antioxidant activity and were mimicked by polyphenolic antioxidants. Because IP formation was Ca^{2+}-dependent, DHPs could have inhibited NT-induced IP formation partly by blocking NT-induced Ca^{2+} influx (Carraway et al., 2003). However, this effect may have been made unimportant by the overriding effects of DHPs on NT binding, which were clearly derived from the antioxidant property. Some of the DHP effect on NT binding (~20%) was reproduced by treatments aimed to perturb Ca^{2+} channel structure (elevation of cellular [Ca^{2+}] and membrane depolarization). Although this might have indicated that Ca^{2+} channels interact with NTR1, the simplest explanation was that these manipulations also acted along the antioxidant pathway.

Based on our finding that the enhancement of NT binding by nifedipine did not require Ca^{2+} and was not reversed by ionomycin, we concluded that DHPs did not act by diminishing Ca^{2+} influx. The direction of the Ca^{2+} flux was also unimportant, because DHP Ca^{2+} channel agonists and antagonists had similar effects. A second hypothesis considered was that Ca^{2+} channel occupation per se was sufficient to promote these effects, because Ca^{2+} channels were known to interact with G proteins (De Waard et al., 1997) and receptors (Grazzini et al., 1996). Postulating that perturbation of VGCC (Catterall, 2000) might alter NTR1 function, we tested the effects of K^+ depolarization and agents known to alter cellular [Ca^{2+}]. The effects observed were relatively small

TABLE 2
Effects of nifedipine on NT binding parameters in Locke and sucrose buffer

Buffer	Agent[a]	Zero Binding[b]	B_{max}[c]	K_i[c]
		cpm/μg	*fmol/mg*	*nM*
Locke	Control	20.4 ± 1.1	165 ± 13	1.0 ± 0.07
Locke	Nifedipine	46.3 ± 3.2[d]	168 ± 11	0.51 ± 0.05[d]
Sucrose	Control	33.7 ± 1.8	165 ± 10	0.61 ± 0.05
Sucrose	Nifedipine	65.8 ± 4.1[d]	175 ± 12	0.35 ± 0.04d

[a] PC3 cells were pretreated 10 min with 50 μM nifedipine or vehicle control. ^{125}I-NT (10^5 cpm, 50 pM) was added and specific binding was measured at 37°C in Locke or sucrose buffer.
[b] Zero binding was defined as specific binding measured at equilibrium in absence of competitor. The results, given as cpm/μg protein, are mean ± S.E.M. from three to six experiments.
[c] K_i was calculated from NT displacement curves generated using 12 concentrations of NT. The results are mean ± S.E.M. for at least three experiments.
[d] Indicates significant difference ($p < 0.05$) compared with appropriate control.

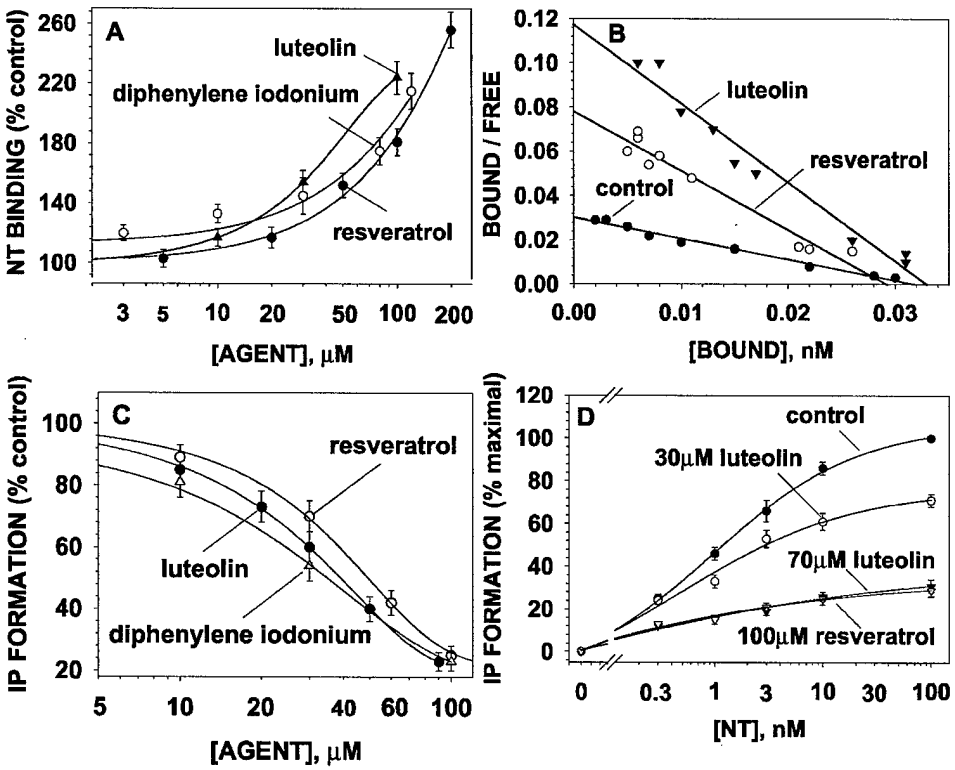


Fig. 6. Antioxidants enhanced NT binding (A) by increasing NTR affinity, not NTR number (B), and they inhibited NT-induced IP formation (C) by decreasing NT efficacy (D). A, PC3 cells were pretreated 10 min with agents indicated and NT binding to PC3 cells was measured. NT binding was significantly ($p < 0.05$) elevated above control for luteolin (>10 μM), resveratrol (>20 μM), and diphenylene iodonium (>3 μM). B, Scatchard plots show that 60 μM luteolin and 150 μM resveratrol increased NTR affinity. The K_i for NT was (nanomolar) 1.07 (control), 0.28 (luteolin), and 0.36 (resveratrol). There was little effect on NTR number (femtomoles per millgram): 170 (control), 175 (luteolin), and 153 (resveratrol). C, PC3 cells were pretreated 10 min with agents indicated, and IP formation in response to 30 nM NT was measured. For the control, NT increased IP formation ~4-fold. IP formation was significantly different from control for luteolin (>10 μM), resveratrol (>30 μM), and diphenylene iodonium (>30 μM). D, PC3 cells were pretreated 10 min with agents indicated, and the dose response for NT-induced IP formation was measured. Luteolin and resveratrol decreases efficacy, i.e., shifted the curves downward. At [NT] >0.3 nM, IP formation was significantly different from the control for each treatment. Results in A, C, and D were from three experiments each. Results in C were from typical experiment that was repeated twice.

and it was unlikely that the far more robust responses to DHPs could be explained on this basis. Therefore, we hypothesized that DHPs altered NTR1 function by mechanism(s) not necessarily involving Ca^{2+} channels.

DHPs are commonly used at concentrations as high as 10 μM to block VGCC, although they are specific for this purpose only in the nanomolar range (Triggle, 2003). Above 1 μM, DHPs disrupt SOCC (Harper et al., 2003), Na^+ channels (Yatani et al., 1988), and K^+ channels (Hatano et al., 2003) and inhibit lipid peroxidation (Diaz-Araya et al., 1998). All of these were possible targets for the effects observed here, given that the IC_{50} value for inhibition of NT-induced IP formation ranged from ~1 μM (felodipine) to ~15 μM (nifedipine). Because Na^+ was known to inhibit NT binding to cell membranes (Carraway et al., 1993), we tested the hypothesis that DHPs enhanced NT binding by blocking Na^+ channels. When sucrose was substituted for NaCl, NT binding was enhanced, but the effects of nifedipine persisted. In agreement with the binding data, NT was more potent in promoting IP formation in the absence of Na^+; however, this had no effect on the ability of nifedipine to inhibit the response to NT. This work and similar studies with K^+ indicated that Na^+ and K^+ were not involved in the effects of nifedipine on NTR1 function, although a conformational coupling involving Na^+ or K^+ channels was still possible.

DHPs are antioxidants that inhibit lipid peroxidation and

TABLE 3. Activity of DHPs and polyphenols on NT binding and NT-induced IP formation

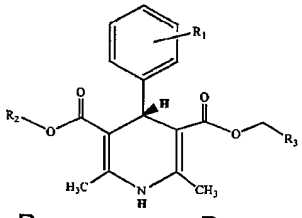
Classification	Agent	NT Binding[a] EC_{50}	IP Formation[b] IC_{50}	Antioxidant[c] Activity
		μM		*Relative*
VGCC antagonist	Felodipine	3	1	2.86
	Nitrendipine	7	2	1.34
	Nicardipine	7	3	
	Nimodipine	7	6	2.12
	Nifedipine	15	15	0.73
	Compound 1[d]	75	28	<0.30
VGCC agonist	(−)-BayK-8644	16	15	
	FPL-64176	29	27	
Antioxidant	Luteolin	40	38	
	Resveratrol	80	48	
	Diphenylene iodonium	61	35	
	BHA	110	N.D.	

N.D., not determined.
[a] EC_{50} was defined as the [agent] giving 75% increase in NT binding. The data are means determined in at least three experiments.
[b] IC_{50} was defined as the [agent] giving 50% decrease in IP formation. The data are means from at least three experiments.
[c] Relative activity coefficient for reactivity to superoxide ion. Data from Ortiz et al. (2003).
[d] Compound 1 (Ortiz et al, 2003) is an *N*-ethyl DHP (structure in Fig 7) that displays reduced reactivity to superoxide. Its effects on calcium channels are not known.

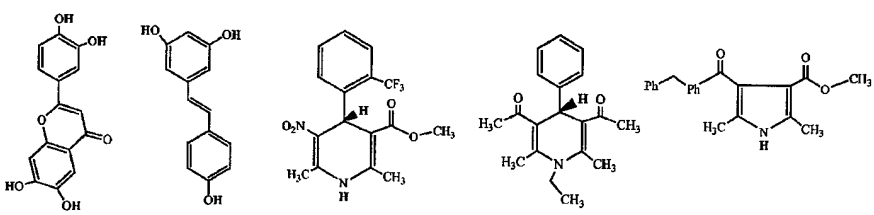
impart cytoprotective effects (Mak et al., 2002). Testing a series of DHPs with known antioxidant ability, we found that the activity order for ability to alter NTR1 function (felodipine > nicardipine > nimodipine > nifedipine) was similar to that reported for inhibition of lipid peroxidation (nicardipine ≅ nimodipine > nifedipine; Diaz-Araya et al., 1998) and for chemical reactivity with superoxide (felodipine > nimodipine > nifedipine; Ortiz et al., 2003). In addition, the effects of DHPs were mimicked by antioxidant polyphenols (luteolin and resveratrol), a hydroxy radical scavenger (BHA), and an inhibitor of flavoprotein oxidases (DPI). The IC_{50} values determined for these agents (Table 3) were in good agreement with values for antioxidant effects in other systems, e.g., luteolin (Hendricks et al., 2003), resveratrol (Leonard et al., 2003) and DPI (Brar et al., 2002). These findings support the hypothesis that DHPs act on NTR1 by a redox-sensitive mechanism, although the target(s) remain to be identified.

For each DHP and antioxidant that enhanced NT binding, there was an associated ability to inhibit NT-induced IP formation. The potency order for these drugs was the same in the two assays (Table 3) and the potency values were correlated ($r^2 = 0.58$), indicating that NT binding and IP formation were similarly sensitive to the chemical properties of these drugs. DHPs might exert two separate effects (one to increase NT binding and another to inhibit IP formation), each having the same drug dependence. Alternatively, they could exert one effect (e.g., altering the state of NTR1) that determines the ability to bind NT and to activate signaling. It is also possible that by inhibiting IP formation, DHPs produce feedback effects on NT binding. Given that DHPs altered NTR1 function at concentrations near to the blood levels (~0.2 μM) in patients receiving these drugs therapeutically (Palma-Aguirre et al., 1995) and below those used for in vitro work (Lopez et al., 1993; Triggle, 2003), these effects could be of clinical and pharmacological importance.

There are multiple mechanisms by which antioxidants might alter NTR1 function. Because NT binding to cell membranes is sulfhydryl-dependent, antioxidants might enhance



	R_1	R_2	R_3
FELODIPINE	(o,m)-Cl_2	-CH_2-CH_3	-CH_3
NICARDIPINE	(m)-NO_2	-$(CH_2)_2$-N-CH_3-CH_2-Ph	-CH_3
NITRENDIPINE	(m)-NO_2	-CH_2-CH_3	-CH_3
NIMODIPINE	(m)-NO_2	-$(CH_2)_2$-O-CH_3	-CH-$(CH_3)_2$
NIFEDIPINE	(o)-NO_2	-CH_3	-CH_3



Luteolin **Resveratrol** **Bay K-8644** **Compound-1** **FPL-64176**

Fig. 7. Chemical structures of DHPs and polyphenols. The basic structure of DHP is shown at the top with the specific substituents for each Ca^{2+} channel blocker. Hydrogens at the 1 and 4 positions (bold) are potential donors. The activity order for effects on NTR function (felodipine > nicardipine ≅ nitrendipine > nimodipine > nifedipine) may relate to the ability of R1 substituents to support hydrogen donation: Cl_2 (felodipine) > meta NO_2 (nitrendipine, nicardipine, and nimodipine) > ortho NO_2 (nifedipine). Polyphenols (bottom left) display less activity than DHPs, due to the less acidic nature of OH versus NH. The activity order (luteolin > resveratrol) may relate to the number of OH-groups (4 versus 3) and conjugated double bonds (8 versus 7). Ca^{2+} channel agonist (−)-BayK-8644 (ortho CF_3 at R1) displays activity like nifedipine (ortho NO_2 at R1). Compound 1 (*N*-ethyl instead of NH) has only one hydrogen donor and displays 20 to 50% activity relative to nifedipine. Ca^{2+} channel agonist FPL-64176 (pyrrole instead of DHP) has only one hydrogen donor and displays ~40% activity relative to nifedipine.

NT binding by maintaining essential sulfhydryl groups in a reduced state. However, this hypothesis is not supported by our finding that cell-permeable sulfhydryl reducing agents (DTT and *N*-acetyl-cysteine) have little effect on NTR1 function and do not interfere with responses to nifedipine. A second possibility is that antioxidants disrupt signaling cascades that modulate NTR1 activity, for example, by protein phosphorylation. Phosphorylation of NTR1 can desensitize the receptor (Hermans and Maloteaux, 1998), and inhibition of this process might give enhanced binding. In some systems, phosphorylation of $G\alpha_q$ is required for activation of PLC (Umemori et al., 1997). Inhibition of this process might diminish NT-induced IP formation. A third idea is that antioxidants might disrupt mitochondrial ATP production, causing secondary effects on GTP/GDP exchange or on kinases involved in NTR1 action.

Although the targets for the actions of DHPs on NTR1 function are not known, the scavenging of ROS is one possibility. Major sources of ROS include mitochondrial enzymes involved in oxidative metabolism (Kamata and Hirata, 1998) and plasma membrane NAD(P)H oxidases involved in signaling by tyrosine kinase receptors (Bae et al., 1997) and G protein-coupled receptors (Seshiah et al., 2002). Thus, ROS scavengers not only protect against oxidative injury but also they inhibit signal transduction involved in inflammation and cell growth (Lassegue and Clempus, 2003). Because NT can induce inflammation and regulate cell growth (Seethalakshmi et al., 1997), it is possible that NT signaling involves ROS. Preliminary data shows that DHPs and polyphenols inhibit NT-induced DNA synthesis (S. Hassan and R. E. Carraway, unpublished data). It also may be important that PKC, which mediates some NT effects (Vincent et al., 1999), is implicated in the activation of NAD(P)H oxidases (Brodie and Blumberg, 2003). Because polyphenolic antioxidants can inhibit protein kinase C (Ferriola et al., 1989), one can imagine multiple mechanisms by which these drugs might disrupt NTR1 function.

The chemical structures of DHPs and polyphenols contain two aromatic ring systems with a number of conjugated double bonds and redox reactive NH or OH group(s) (Fig. 7). These features seem to be essential for this activity, given that equally powerful antioxidants lacking these structures are totally ineffective. Notable is the inactivity of α-tocoferol, which is a standard for many assays (Mitchell et al., 1998). In general, DHPs are more potent than polyphenols in altering NTR1 function, and this may be related to the acidity of the hydrogen donor (NH > OH). The activity of compounds within each group also varies (Table 3), and this might be due to the influence of phenyl ring substituents on donor acidity and on resonance stabilization of reaction intermediates (Solomons, 1994). Another important determinant of reactivity could be the membrane partition coefficient (Mason et al., 1999).

The chemical structures of DHPs are reminiscent of NADH. The reaction scheme whereby NAD-linked dehydrogenases donate hydrogen atoms to substrates is shown in Fig. 8A. One hydrogen is transferred from NADH as a hydride ion (H^-) and another is taken as H^+ from the medium (Lehninger, 1982). It is tempting to speculate that DHPs can react analogously, transferring hydrogen atoms to superoxide by way of cationic (Fig. 8B) or radical intermediates (Fig. 8C) to generate pyridine derivatives and water. DHPs are known to form pyridine adducts when reacted with alkyl radicals (Nunez-Vergara et al., 2003). Because the stability of the intermediates in Fig. 8 is negatively affected by electron withdrawal, this predicts that nitro groups in the phenyl ring (especially ortho) would diminish reactivity. The order derived from such considerations (felodipine > nitrendipine ≅ nicardipine ≅ nimodipine > nifedipine) is in fair agreement with that measured by Ortiz et al. (2003) and that found here

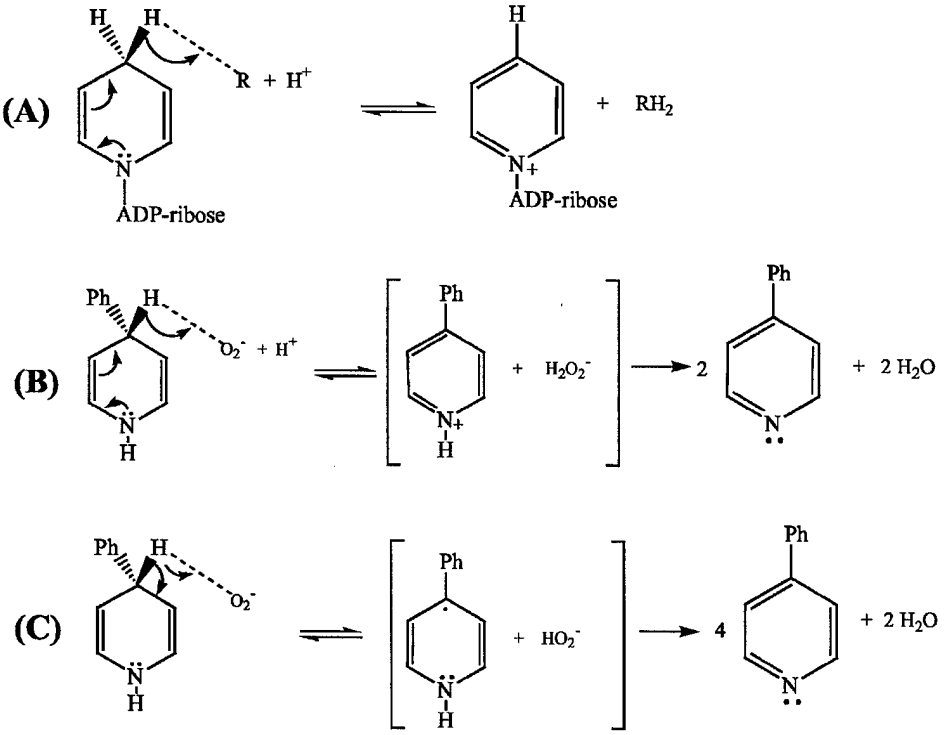


Fig. 8. Reaction scheme for NAD-linked dehydrogenase action (A) compared with those proposed for reaction of DHPs with superoxide anion (B and C). In A, NADH is oxidized to NAD^+ as it donates a hydride ion (H^-) to acceptor (R), which reacts with H^+ to give RH_2. In B, we propose an analogous two-electron transfer reaction, whereby DHP donates H^- to superoxide anion (O_2^-) forming a pyridinium cation intermediate that further donates H^+ to the peroxide anion. In C, a single electron transfer is shown forming a neutral radical intermediate. In both cases, a pyridine derivative and water are the final products. For simplicity, we show the dihydropyridine ring with the attached phenyl group (Ph), but the various substituents are omitted.

for altering NTR1 function. Because nitrendipine, nicardipine, and nimodipine each have nitro in the meta position, a near equal reactivity with superoxide is expected. The differences in their activity in our system might be attributed to the effects of other ring substituents on lipophilicity (Fig. 7), which could affect their ability to enter cells and partition into membranes.

DHPs inhibit cardiac contractility and relax vascular smooth muscle, and their relative abilities to do so vary >10-fold. Felodipine is ~10-times more vascular-selective than nifedipine (Triggle, 2003). Although this might be due to differential expression of various Ca^{2+} channels, it is tempting to speculate that the antioxidative effects of DHPs also contribute. For example, if the relaxant effects of DHPs on vascular smooth muscle involve ROS signaling, this might explain the enhanced activity of felodipine relative to nifedipine.

In conclusion, DHPs enhance NT binding and inhibit NT-induced IP formation by an indirect mechanism that seems to require an aromatic structure and functional groups to facilitate hydrogen atom donation. For a series of DHPs, the ability to alter NTR1 function correlates to the ability to scavenge superoxide anion. Polyphenolic antioxidants and an inhibitor of flavoprotein oxidases mimic the effects of DHPs. We propose that DHPs disrupt NTR1 function by inhibiting cellular oxidative reaction(s) or by scavenging ROS involved in receptor regulation and signal transduction.

Acknowledgments

We thank undergraduates Li Ming Tseng and Amy Wu for laboratory work and Ann Przyborowska for help with graphics. We thank Juan Arturo Squella (Pharmaceutical Sciences, University of Chile) for the generous gift of compound-1.

References

Bae Ys, Kang SW, Seo MS, Baines IC Tekle E, Chock PB, and Rhee SG (1997) Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* **272:**217–221.

Belmeguenai A, Leprince J, Tonon MC, Vaudry H, and Louiset E (2002) Neurotensin modulates the amplitude and frequency of voltage-activated Ca^{2+} currents in frog pituitary melanotrophs: implication of the inositol triphosphate/protein kinase C pathway. *Eur J Neurosci* **16:**1907–1916.

Brar SS, Kennedy TP, Sturrock AB, Huecksteadt TP, Quinn MT, Murphy TM, Chitano P, and Hoidal JR (2002) NADPH oxidase promotes NF-κB activation and proliferation in human airway smooth muscle. *Am J Physiol* **282:**L782–L795.

Brodie C and Blumberg PM (2003) Regulation of cell apoptosis by protein kinase C delta. *Apoptosis* **8:**19–27.

Carraway R and Leeman SE (1976) Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. *J Biol Chem* **251:**7045–7052.

Carraway RE, Gui X, and Cochrane DE (2003) Ca^{2+}-channel blockers enhance neurotensin (NT) binding and inhibit NT-induced inositol phosphate formation in prostate cancer PC3 cells. *J Pharm Exp Ther* **307:**640–650.

Carraway RE, Mitra SP, and Honeyman TW (1993) Effects of GTP analogs and metal ions on the binding of neurotensin to porcine brain membranes. *Peptides* **14:**37–45.

Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* **16:**521–555.

De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, and Cambell KP (1997) Direct binding of G-protein βγ complex to voltage-dependent calcium channels. *Nature (Lond)* **385:**446–450.

Diaz-Araya G, Goday L, Naranjo L, Squella JA, Letelier ME, and Nunez-Vergara LJ (1998) Antioxidant effects of 1,4-dihydropyridine and nitroso aryl derivatives on the Fe^{+3}/ascorbate-stimulated lipid peroxidation in rat brain slices. *Gen Pharmacol* **31:**385–391.

Ferriola PC, Cody V, and Middleton E Jr (1989) Protein kinase C inhibition by plant flavonoids: kinetic mechanism and structure activity relationships. *Biochem Pharmacol* **38:**1617–1624.

Ferris CF (1989) Neurotensin, in *Gastrointestinal System II* (Schultz SG and Makhlouf GM eds) pp 559–586, Oxford University Press, New York.

Gailly P (1998) Ca^{2+}-entry in CHO cells, after Ca^{2+} stores depletion, is mediated by arachidonic acid. *Cell Calcium* **24:**293–304.

Golba KS, Deja M, Imiolek P, Kotyla PJ, Biernat J, Wos S, and Herman ZS (1995) The dihydropyridines modulate neurotensin inotropic action paradoxically. *J Physiol Pharmacol* **46:**419–427.

Grazzini E, Durroux T, Payet MD, Bilodeau L, Gallo-Payet N, and Guillon G (1996) Membrane-delimited G protein-mediated coupling between V1a vasopressin receptor and dihydropyridine binding sites in rat glomerulosa cells. *Mol Pharmacol* **50:**1273–1283.

Harper JL, Camerini-Otero CS, Li A, Kim S, Jacobson KA, and Daly JW (2003) Dihydropyridines as inhibitors of capacitative calcium entry in leukemic HL-60 cells. *Biochem Pharmacol* **65:**329–338.

Hatano N, Ohya S, Muraki K, Giles W, and Imaizumi Y (2003) Dihydropyridine Ca2+ channel antagonists and agonists block Kv4.2, Kv4.3 and Kv1.4 K+ channels expressed in HEK293 cells. *Br J Pharmacol* **139:**533–544.

Hendricks JJ, de Vries HE, van der Pol SMA, van den Berg TK, van Tol EAF, and Dijkstra CD (2003) Flavonoids inhibit myelin phagocytosis by macrophages; a structure-activity relationship study. *Biochem Pharmacol* **65:**877–885.

Hermans E and Maloteaux JM (1998) Mechanisms of regulation of neurotensin receptors. *Pharmacol Ther* **79:**89–104.

Janero DR and Burghardt B (1989) Antiperoxidant effects of dihydropyridine calcium antagonists. *Biochem Pharmacol* **38:**4344–4348.

Kamata H and Hirata H (1998) Redox regulation of cellular signalling. *Cell Signal* **11:**1–14.

Lassegue B and Clempus RE (2003) Vascular NAD(P)H oxidases: specific features, expression and regulation. *Am J Physiol* **285:**R277–R297.

Lehninger AL (1982) Electron transport, oxidative phosphorylation and regulation of ATP production, in *Principles of Biochemistry* (Anderson S and Fox J eds) pp 467–510, Worth Publishers, New York.

Leonard SS, Xia C, Jiang BH, Stinefelt B, Klandorf H, Harris GK, and Shi X (2003) Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem Biophys Res Commun* **309:**1017–1026.

Lopez MG, Fonteriz RI, Gandia I, de la Fuente M, Villarroya M, Garcia Sanchl B, and Garcia AB (1993) The nicotinic acetylcholine receptor in bovine chromaffin cell, a new target for dihydropyridines. *Eur J Pharmacol* **247:**199–207.

Mak IT, Zhang J, and Weglicki WB (2002) Protective effects of dihydropyridine Ca^{2+}-blockers against endothelial cell oxidative injury due to combined nitric oxide and superoxide. *Pharmacol Res* **45:**27–33.

Mason RP, Mak IT, Trumbore MW, and Mason PE (1999) Antioxidant properties of calcium antagonists related to membrane biophysical interactions. *Am J Cardiol* **84:**16L–22L.

Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, and Duthie GG (1998) Antioxidant efficacy of phytoestrogens in chemical and biological model systems. *Arch Biochem Biophys* **360:**142–148.

Mitra SP and Carraway RE (1993) Importance of thiol group(s) in the binding of ^{125}I-labeled neurotensin to membranes from porcine brain. *Peptides* **14:**185–189.

Mule F and Serio R (1997) Mode and mechanism of neurotensin action in rat proximal colon. *Eur J Pharmacol* **319:**269–272.

Nunez-Vergara LJ, Lopez-Alarcon C, Navarrete-Encina PA, Atria AM, Camargo C, and Squella JA (2003) Electrochemical and EPR characterization of 1,4-dihydropyridines. Reactivity towards alkyl radicals. *Free Radic Res* **37:**109–120.

Ortiz ME, Nunez-Vergara LJ, and Squella JA (2003) Relative reactivity of dihydropyridine derivatives to electrogenerated superoxide ion in DMSO solutions: a voltametric approach. *Pharm Res (NY)* **20:**292–296.

Palma-Aguirre JA, Montoya-Cabrera MA, du Souich P, Hoyo-Vadillo C, Flores-Murrieta FJ, and Castaneda-Hernandez G (1995) Discrepancy between bioavailability and hypotensive effect of oral and sublingual nifedipine. *Am J Ther* **2:**3–9.

Rice-Evans CA, Miller NJ, and Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20:**933–956.

Rostene WH and Alexander MJ (1997) Neurotensin and neuroendocrine regulation. *Front Neuroendocrinol* **18:**115–173.

Seethalakshmi L, Mitra SP, Dobner PR, Menon M, and Carraway RE (1997) Neurotensin receptor expression in prostate cancer cell line and growth effect of NT at physiological concentrations. *Prostate* **31:**183–192.

Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, and Griendling KK (2002) Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* **91:**406–413.

Solomons TWG (1994) Electrophilic aromatic substition, in *Fundamentals of Organic Chemistry*, 4th ed., pp 599–641, John Wiley & Sons, New York.

Triggle DJ (2003) The 1,4-dihydropyridine nucleus: a pharmacophoric template part 1. Actions at ion channels. *Mini Rev Med Chem* **3:**217–225.

Trudeau LE (2000) Neurotensin regulates intracellular calcium in ventral tegmental area astrocytes: evidence for the involvement of multiple receptors. *Neuroscience* **91:**293–302.

Umemori H, Inoue T, Kume S, Sekiyama N, Nagao M, Itoh H, Nakanishi S, Mikoshiba K, and Yamamoto T (1997) Activation of the G protein $G_{q/11}$ through tyrosine phosphorylation of the α subunit. *Science (Wash DC)* **276:**1878–1881.

Vincent JP, Mazella J, and Kitabgi P (1999) Neurotensin and neurotensin receptors. *Trends Pharmacol Sci* **20:**302–309.

Yatani A, Kunze DL, and Brown AM (1988) Effects of dihydropyridine calcium channel modulators on cardiac sodium channels. *Am J Physiol* **254:**H140–H147.

Zheng W, Rampe D, and Triggle DJ (1991) Pharmacological, radioligand binding, and electrophysiological characteristics of FPL-64176, a novel nondihydropyridine Ca^{2+} channel activator, in cardiac and vascular preparations. *Mol Pharmacol* **40:**734–741.

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Regulatory Peptides 120 (2004) 155–166

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Involvement of MAP-kinase, PI3-kinase and EGF-receptor in the stimulatory effect of Neurotensin on DNA synthesis in PC3 cells

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Received 26 December 2003; received in revised form 2 March 2004; accepted 9 March 2004

Available online 21 April 2004

Abstract

The mechanism by which neurotensin (NT) promotes the growth of prostate cancer epithelial cells is not yet defined. Here, androgen-independent PC3 cells, which express high levels of the type 1 NT-receptor (NTR1), are used to examine the involvement of epidermal growth factor receptor (EGFR), mitogen-activated protein kinases (ERK, SAPK/JNK and p38), PI3 kinase and PKC in the mitogenic effect of NT. NT dose dependently (0.1–30 nM) enhanced phosphorylation of EGFR, ERK and Akt, reaching maximal levels within 3 min as measured by Western blotting. These effects were associated with an accumulation of EGF-like substance(s) in the medium (assayed by EGFR binding) and a 2-fold increase in DNA synthesis (assayed by [^{3}H]thymidine incorporation). The DNA synthesis enhancement by NT was non-additive with that of EGF. The NT-induced stimulation of EGFR/ERK/Akt phosphorylation and DNA synthesis was inhibited by EGFR-tyrosine kinase inhibitors (AG1478, PD153035), metallo-endopeptidase inhibitor phosphoramidon and by heparin, but not by neutralizing anti-EGF antibody. Thus, transactivation of EGFR by NT involved heparin-binding EGF (HB-EGF or amphiregulin) rather than EGF. The effects of NT on EGFR/ERK/Akt activation and DNA synthesis were attenuated by PLC-inhibitor (U73122), PKC-inhibitors (bisindolylmaleimide, staurosporine, rottlerin), MEK inhibitor (U0126) and PI3 kinase inhibitors (wortmannin, LY 294002). We conclude that NT stimulated mitogenesis in PC3 cells by a PKC-dependent ligand-mediated transactivation of EGFR, which led to stimulation of the Raf–MEK–ERK pathway in a PI3 kinase-dependent manner.

Keywords: Neurotensin; PC3; ERK; PI3 kinase; EGFR

1. Introduction

Prostate cancer is the most common non-cutaneous malignancy and the second leading cause of death in American males [1]. Patient deaths are usually attributed to disseminated disease that is hormone refractory and resistant to conventional therapies [2]. Androgen ablation therapy, while initially effective in slowing disease progression, eventually fails as androgen-insensitive tumors recur [3]. The factors that support androgen-independent growth and facilitate metastasis are just being uncovered [4–7]. Recent studies have shown the importance of receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), which can stimulate DNA synthesis and initiate cell cycle progression [8,9]. These effects primarily involve the activation of extracellular signal regulated protein kinases (ERK), which translocate to the nucleus and activate transcription factors [10–12]. In late-stage prostate cancers, a common finding is that EGFR and ERK are overexpressed and largely activated, and this can be a prognostic marker for poor clinical outcome [5,6]. In addition, clusters of neuroendocrine cells are frequently found in late-stage tumors and are thought to secrete factors that maintain the tumor in the absence of androgen [13]. Some neuroendocrine peptides—including bradykinin, bombesin and neurotensin (NT)—exert growth-stimulatory effects in prostate cancer cells [14–16] via specific G protein-coupled receptors (GPCRs).

NT is a growth-regulatory peptide found in nerves and endocrine cells throughout the body [17–19], which increases the number and size of carcinogen-induced tumors

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0167-0115/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2004.03.004

[20] and stimulates the growth of cancer cell lines in vitro and in vivo [16,21–23]. High fat intake stimulates NT release [24,25], and epidemeologic studies associate fatty diets with an increased incidence of prostate cancer [26]. The mRNA for the type1 NT receptor (NTR1) is overexpressed in many cancers including prostate [27], and high levels of functional NTR1 are found in late-stage, androgen-independent prostate cancer cells such as PC3 [16]. Biochemical studies indicate that NTR1 is coupled to $G_{q/11}$ in a variety of cell systems [28]. Consistent with this, NT stimulates the rapid formation of inositol phosphates and elevates [Ca^{2+}] in PC3 cells (RE Carraway, unpublished data). Exposure of PC3 cells to NT for 24 h stimulates DNA synthesis and enhances cell growth [16]. Although the mechanisms involved are not yet defined, the importance of EGF as a growth regulator for these cells suggests that NT may interact with the EGF system. Support for this idea derives from the finding that NT potentiates the mitogenic effect of EGF in primary cultures of rat hepatocytes [29]. These facts led us to hypothesize that the growth-promoting effect of NT in PC3 cells is mediated by the EGF system.

There is a rich and complex literature on growth-enhancement by GPCR agonists, much of which focuses on mechanisms of cross-talk along the EGF signaling pathway [30–33]. In many cell systems, GPCR agonists influence growth by transactivating EGFR [34–36], although there are instances where the GPCR signal inputs downstream at the level of Ras or Raf [37,38]. While transactivation of EGFR can occur by a direct interaction with GPCR [39] or by src-mediated phosphorylation of EGFR [40,41], the predominant mechanism appears to involve protease-mediated liberation of an EGF-like ligand from the cell surface [34–36]. These pathways are very complex but the basic features of the signaling cascade include (a) activation of EGFR tyrosine kinase; (b) Ras-mediated activation of protein kinase Raf; (c) Raf-mediated activation of protein kinase MEK; and (d) MEK-mediated activation of protein kinase ERK. Other inputs to this pathway, which can be critical, include PKC and PI3-kinase. While PKC has been shown to phosphorylate and activate Raf [42], PI3-kinase is purported to assist in the recruitment of Raf to membranes [43], and also might activate specific members of the PKC family [44].

Growth stimulation by NT in pancreatic MIA-PaCa2 cancer cells has been shown to involve ERK activation but the mechanism has not been defined [45]. The effects of NT on the ERK pathway in prostate cancer cells have not been studied. Therefore, the present study was undertaken to define the underlying intracellular signaling mechanisms involved in the mitogenic effect of NT in PC3 cells. Here we provide definitive evidence that NT causes ligand-mediated transactivation of EGFR, leading to PKC-dependent and PI3-kinase-dependent activation of ERK. Furthermore, we show that the activation of this pathway is essential for the mitogenic response to NT.

2. Material and methods

2.1. Cell culture and reagents

Human androgen-insensitive prostate cancer PC3 cells were obtained from the American Type Culture Collection, Rockville, MD and were maintained in the tissue culture facility, UMMS [16]. The F12K medium was supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 1 mM glutamine. Antibodies (phospho-MAPK and MAPK, phospho-akt and akt or phospho-EGFR and EGFR) were obtained from Santa Cruz Biotechnology, CA. Human-specific neutralizing anti-EGF was obtained from Upstate Biotechnology, NY. PKC activator Phorbol-12-myristate-13-acetate (PMA), Ca^{+2} chelators BAPTA/AM and EGTA, Ca^{2+}-calmodulin kinase inhibitor KN62, heparin, EGFR inhibitors tyrphostin AG1478 and PD153035, metallo-endopeptidase inhibitor phosphoramidon, PLC inhibitor U73122, PKC inhibitors staurosporine, bisindolylmaleimide and rottlerin, PI3 kinase inhibitors wortmannin and LY 294002 and MEK1/2 inhibitor U01206 were purchased from Calbiochem (San Diego, CA).

2.2. Cell proliferation assay

The assay was performed as described previously [16,46]. In brief, 10^4 cells were plated per well in 24-well plates. After 24 h, cells were serum-starved 48 h, and fresh medium was added with stimuli. After 24 h, [^{3}H]thymidine (1 μCi/well) was added for 3 h. When used, inhibitors were added 30 min before stimulation. DNA was precipitated with 6% TCA, washed in PBS and ethanol, then solubilized in 0.3 N NaOH–0.1% SDS, and counted by liquid scintillation spectrometry.

2.3. Western blot analysis

Prior to the experiment (24 h), serum was withdrawn from 80% confluent PC3 cells in 60 mm dishes. Cells were washed with hepes-buffered Locke (Locke): 148 mM NaCl; 5.6 mM KCl, 6.3 mM hepes; 2.4 mM $NaHCO_3$; 1.0 mM $CaCl_2$; 0.8 mM $MgCl_2$; 5.6 mM glucose and 0.1% BSA. Then, cells were exposed to stimuli at 37 °C and reactions were stopped by washing with ice-cold PBS with inhibitors: 50 mM NaF, 2 mM Na_3 VO_4, 5 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM PMSF, 1 mM *o*-phenanthroline. Cells were placed on ice and scraped into 2 × SDS buffer with inhibitors. Samples were sonicated on ice for 20 s and centrifuged 30 min at 30,000 × *g*. Equal amounts of protein (≅30 μg) were separated by SDS-PAGE on 10% polyacrylamide minigels. Proteins were electroeluted onto PVDF (Immobilon P, Millipore). After blocking in 5% non-fat dry milk in TTBS: 0.05% Tween 20, 20 mM Tris, 0.5 M NaCl for 1 h and washing 3X with TTBS, blots were incubated with the primary anti-

serum in blocking buffer for 18 h at 4 °C. Blots were washed in TTBS, then incubated with horseradish peroxidase-linked secondary antibody for 1 h at 20 °C, and washed again with TTBS. Enhanced chemiluminescence was performed according to manufacturer (Santa Cruz) and autoradiographs were scanned for computerized densitometric analysis. After staining with one antibody, some blots were stripped and reprobed using a different antibody for comparison and for normalization. Stripping was performed with 62.5 mM Tris–HCl, 2% SDS, 0.1 M β-mercaptoethanol, pH 6.8 for 1 h at 70 °C, followed by washing with TBS.

2.4. EGF iodination and binding to PC3 cells

EGF was iodinated using choloramine T and the monoiodinated product was purified by HPLC to a specific activity of ≅2000 Ci/mmol [16]. The μ-Bondapak C18 column was eluted at 1.5 ml/min using a linear gradient from 0.1% trifluroacetic acid to 50% CH_3CN. PC3 cells in 24-well plates were washed with Locke, and EGF binding was performed at 37 °C for 25 min or at 4°C for 3 h, using 10^5 cpm/ml ^{125}I-labeled EGF in 1.0 ml Locke [47]. Human EGF (0.05–10 nM) was added to construct a standard curve and Sep-Pak concentrated PC3-conditioned media were assayed. The conditioned media were collected from control or NT treated subconfluent serum-starved PC3 cells. Media were adsorbed to methanol-activated Sep-Paks (Waters, Milford, MA), washed with water, eluted with 75% CH_3CN, and lyophilized. Cell binding was stopped on ice, the medium was aspirated and cells were washed three times in 2 ml ice-cold saline. Dissociation was less than 1% during binding.

2.5. Statistical analysis

All experiments were repeated at least three times with similar results. The experimental values obtained were expressed as mean ± S.E.M. with N indicating the number of independent observations. Statistical analysis was performed with the graph-pad prism. Comparisons with $p<0.05$ were considered statistically significant.

3. Results

3.1. NT-induced and EGF-induced DNA synthesis were non-additive

DNA synthesis in PC3 cells was stimulated dose-responsively by NT (EC50, ≅0.3 nM) and by EGF (EC50, ≅0.1 nM), each giving a maximal enhancement of ≅2-fold (Fig. 1A). The effect of NT and EGF in combination was less than additive (Fig. 1B). These results suggested that NT and EGF signal transmission involved a common pathway.

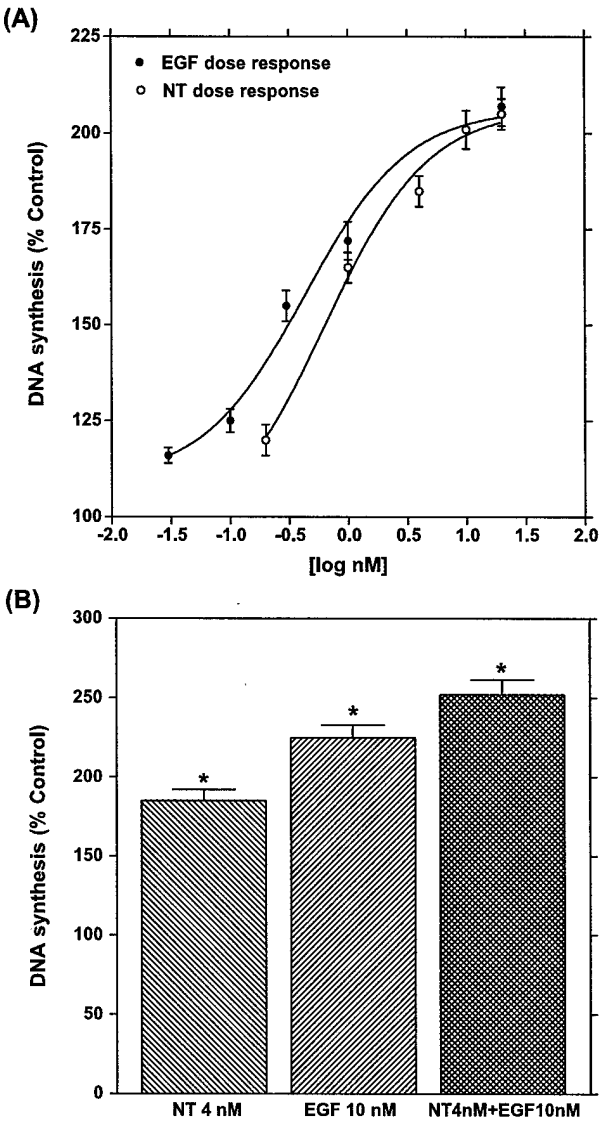

Fig. 1. Dose–response relationships (A) and non-additivity (B) for the effects of NT and EGF on DNA synthesis in PC3 cells. Quiescent PC3 cells were stimulated for 24 h with various doses of NT or EGF, and [^{3}H]thymidine incorporation into DNA was measured during the final 3 h. (A) The log dose–response plots show a 2-fold stimulation of DNA synthesis by NT (EC50, ≅0.5 nM) and by EGF (EC50, ≅0.3 nM). Results are from 6 experiments performed in triplicate. (B) Bar graph plots show that maximal doses of NT (4 nM) and EGF (10 nM) increased DNA synthesis ≅1.9-fold and ≅2.2-fold, respectively. The combined effect was less than additive (≅2.5-fold). Results were from three experiments performed in triplicate.

3.2. NT stimulated phosphorylation of ERK, Akt and EGFR

Some growth responses to EGF involve activation of p42 and p44 extracellular regulated kinases (ERK) by specific phosphorylation events [6–9]. To assess the involvement of this pathway in the growth stimulatory effect of NT, we performed Western blotting using antisera specific to ERK and phosphorylated ERK. Stimulation of PC3 cells with 20 nM NT caused a rapid, transient phosphorylation of ERK

that reached a maximum (≅12-fold enhancement) at 3 min and then declined without a change in the total amount of ERK (Fig. 2A). The dose–response relationship (Fig. 3A) gave EC50≅0.8 nM, which was in fair agreement with that for the effect of NT on DNA synthesis.

PI3-kinase, another important input into cell growth and survival, has effects that involve downstream activation of the protein kinase Akt [48,49]. Western blotting, using antisera specific to Akt and phosphorylated Akt, was employed to assess the involvement of this pathway in the growth effect of NT. Stimulation of PC3 cells with NT enhanced (up to 3-fold) the phosphorylation of Akt in a time-dependent (Fig. 2B) and dose-dependent (Fig. 3B) manner without altering the total amount of Akt.

Since EGF can stimulate ERK and PI3-kinase by activating EGFR tyrosine kinase and since some GPCR agonists transactivate EGFR, we postulated that NT activated ERK and PI3-kinase by transactivating EGFR. Western blotting, using antisera specific to EGFR and phosphorylated EGFR, was employed to assess the involvement of EGFR in the response to NT. Stimulation with NT increased phosphorylation of EGFR without altering the total EGFR content. Although the dose–response relationship (Fig. 3C) was similar to that for activation of ERK and Akt, the time-course (Fig. 2C) indicated that activation of EGFR preceded the other responses. These results indicated that NT caused the activation of EGFR, which may have secondarily activated ERK and PI3-kinase.

In contrast, NT did not affect the phosphorylation of p38 or SAPK/JNK when used at the same doses and assayed at the same times (results not shown).

3.3. The effects of NT on EGFR, ERK and DNA synthesis were blocked by EGFR tyrosine kinase inhibitors, metallo-endopeptidase inhibitor and heparin

To test the hypothesis that EGFR was an essential participant in the stimulatory effects of NT on ERK and DNA synthesis, we examined the effects of specific tyrosine kinase inhibitors. AG1478 and PD153035 dose dependently

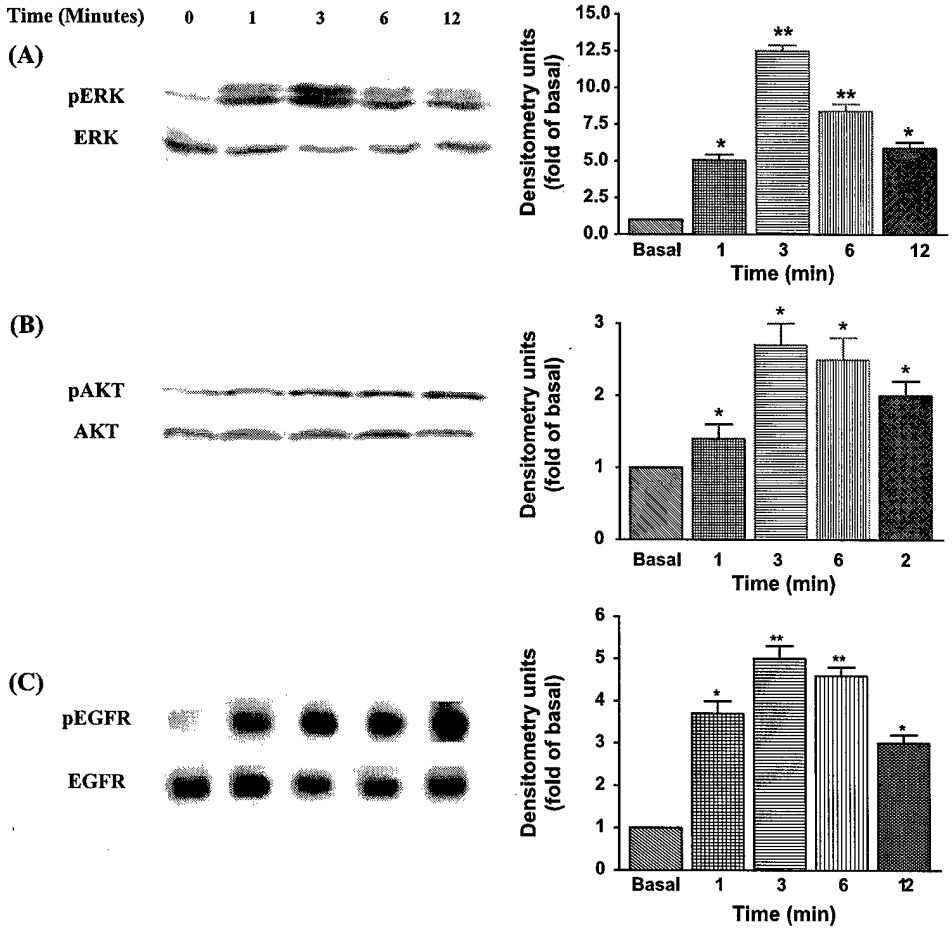

Fig. 2. Time course of NT-stimulated activation of ERK (A) Akt (B) and EGFR (C) in PC3 cells. Quiescent PC3 cells were stimulated with 20 nM NT for 1 to 12 min. Cell lysates were subjected to Western blotting using phospho-specific and enzyme-specific antibodies to (A) ERK; (B) Akt; and (C) EGFR. NT caused a rapid, transient phosphorylation of ERK, Akt and EGFR that reached a maximum at 3 min and then declined. Activation of EGFR preceded the other responses. Results were from three experiments. *$p<0.05$; **$p<0.01$ as compared to the basal level.

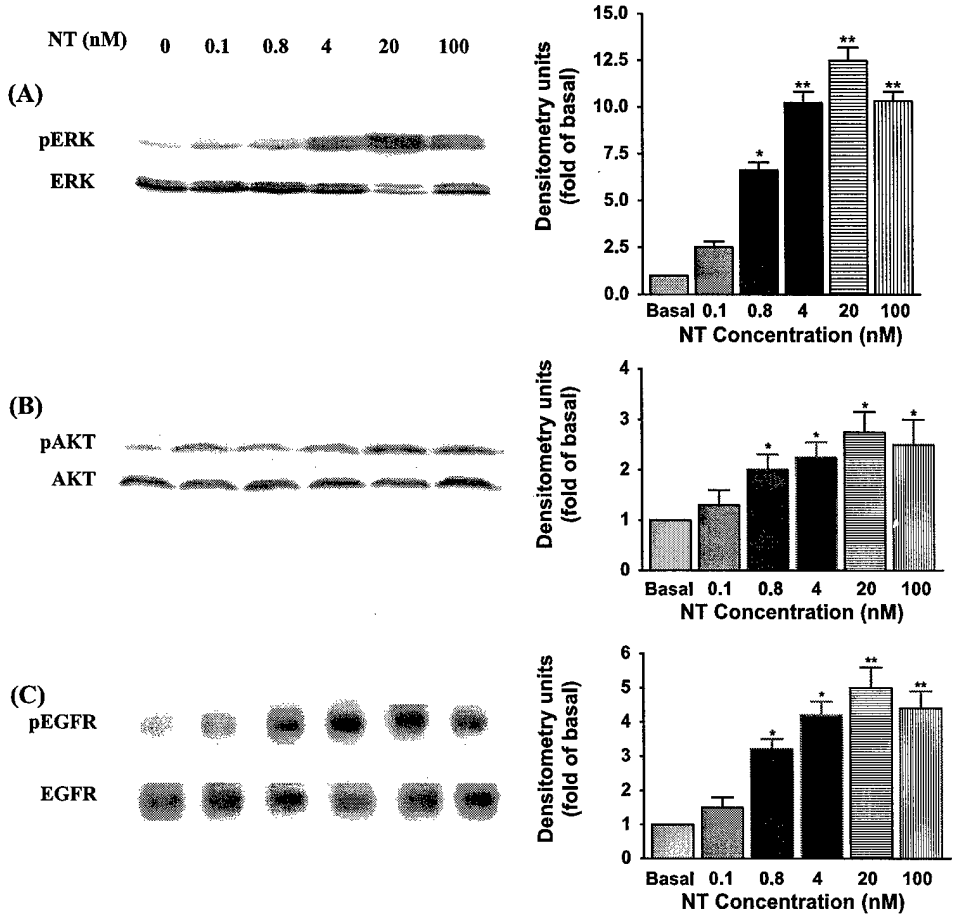

Fig. 3. Concentration-dependence of NT-stimulated activation of ERK (A) Akt (B) and EGFR (C) in PC3 cells. Quiescent PC3 cells were stimulated with various concentrations of NT for 3 min. Cell lysates were subjected to Western blotting using phospho-specific and enzyme-specific antibodies to (A) ERK; (B) Akt; and (C) EGFR. NT enhanced the phosphorylation of ERK, Akt and EGFR in a dose-dependent manner with an EC50 ≅ 0.8 nM. Results were from three experiments. $*p<0.05$; $**p<0.01$ as compared to the basal level.

inhibited NT-induced activation of EGFR and ERK (Fig. 4A) and blocked the effect of NT on DNA synthesis (Fig. 4B), indicating that activation of EGFR was required for these effects of NT.

Activation of metalloproteinases and subsequent transactivation of EGFR by GPCR agonists was reported [36]. In our experiment, we found that phosphoramidon a metalloendopeptidase inhibitor blocked the NT-induced activation of EGFR, Akt and ERK (Fig. 5A) and significantly inhibited the effect of NT on DNA synthesis (Fig. 5B). However, phosphoramidon did not alter these effects of EGF (Fig. 5A,B).

Activation of EGFR in response to certain GPCR agonists was reported to involve the liberation of heparin-binding (HB)-EGF [34–36], the effects of which could be blocked by heparin, a competitive inhibitor acting specifically at the proteoglycan coreceptor [50]. In the present study, heparin attenuated NT-induced activation of EGFR, Akt and ERK (Fig. 6A). Heparin also blocked the effect of NT on DNA synthesis (Fig. 6B). In contrast, neutralizing anti-EGF did not alter these effects of NT, although it diminished the response to EGF (Fig. 6A). Taken together, these results suggested that NT stimulated ERK phosphorylation and DNA synthesis by activating EGFR via release of the ligand HB-EGF.

3.4. NT stimulated accumulation of EGF-like material in the medium

To further examine the involvement of HB-EGF in the response to NT, we measured EGF-like material in PC3 cell conditioned media using a cell-based radioreceptor assay. The EGF-like substances were Sep-Pak concentrated and assayed for ability to displace ^{125}I-EGF from binding sites on PC3 cells in vivo. Incubation of PC3 cells with 20 nM NT caused an accumulation of EGF-like material in the media (Fig. 7), the levels of which were significantly elevated above control at 24 h (≅ 75% increase) and 48 h (≅ 125% increase). Although the concentration of EGF-like material in the media was in the pM range, which was below that

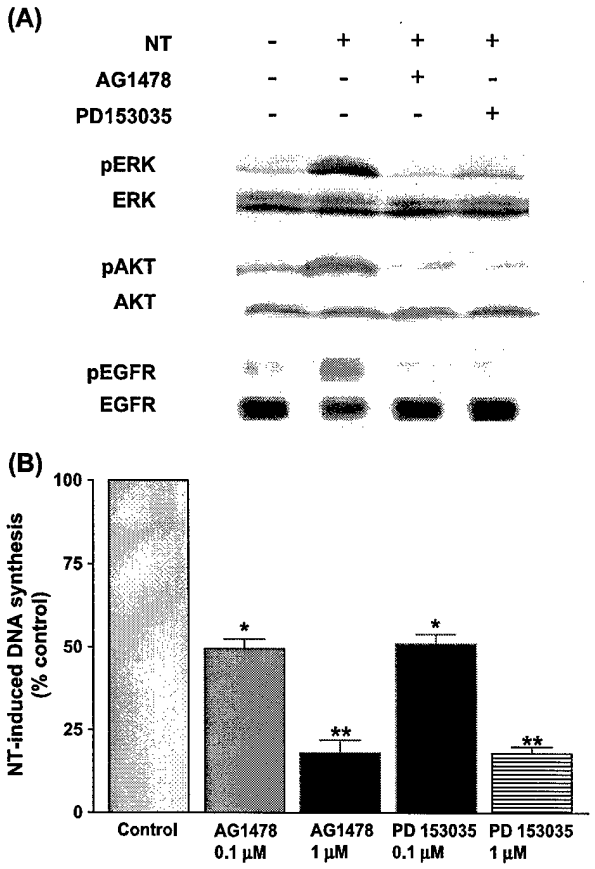

Fig. 4. Effects of EGFR-specific tyrosine kinase inhibitors (AG1478, PD153035) on phosphorylation of ERK, Akt and EGFR (A) and DNA synthesis (B) in response to NT. In (A), PC3 cells were preincubated with AG1478 (1 μM) or PD153035 (1 μM) for 10 min and then stimulated with NT (20 nM) for 3 min. AG1478 and PD153035 blocked NT-induced activation of ERK, Akt and EGFR. This experiment was performed three times with similar results. In (B), PC3 cells were pretreated with each inhibitor (0.1 and 1.0 μM) for 30 min, prior to assessing the effect of 20 nM NT on DNA synthesis. AG1478 and PD153035 dose dependently inhibited NT-induced DNA synthesis. Results were from six experiments performed in triplicate. $*p<0.05$; $**p<0.01$ as compared to the control.

required for EGFR activation, it was likely that considerably more EGF-like material was bound to EGFR. These results suggested that NT enhanced the release of EGF-like material (HB-EGF), which secondarily activated EGFR.

3.5. NT-induced growth effects involved PLC but were CA^{2+}-independent

Previously [16], we showed that NT-induced DNA synthesis and growth in PC3 cells were mediated by type1 NT receptor (NTR1), since they were blocked by antagonist SR48692. NTR1 is a $G_{q/11}$-coupled receptor that activates PLC, enhances inositol phosphate formation and elevates cellular $[Ca^{2+}]$ in response to NT. To determine the involvement of PLC and cellular $[Ca^{2+}]$ in the effects of NT on EGFR, ERK and DNA synthesis, cells were treated with specific inhibitors prior to testing. PLC inhibitor U73122 attenuated the effects of NT on EGFR, Akt and ERK (Fig. 8A), as well as on DNA synthesis (Fig. 8B). To confirm the involvement PLC and its substrate PIP2, we tested the effects of quercetin, which diminishes PIP2 levels by inhibiting PI4-kinase and PI5-kinase [51]. Quercetin inhibited NT-induced ERK phosphorylation measured at 3 min (IC50, ≅20 μM) and NT-induced DNA synthesis measured at 24 h (IC50, ≅1 μM).

In contrast, Ca^{2+}-chelators (EGTA, BAPTA-AM) and Ca^{2+}/calmodulin kinase inhibitor KN62 did not impair NT-induced activation of EGFR, Akt and ERK (Fig. 8A), although $Ca^{2+}$was necessary for NT-induced DNA synthesis (Fig. 8B). These results indicated that although NT's effects were mediated by PLC, the Ca^{2+}-elevation following

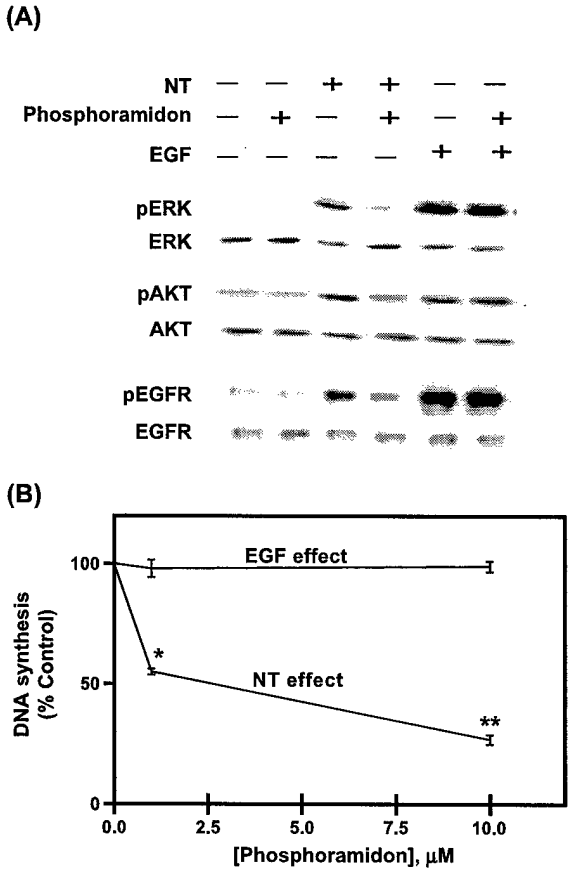

Fig. 5. Effect of metallo-endopeptidase inhibitor phosphoramidon on phosphorylation of ERK, Akt and EGFR (A) and DNA synthesis (B) in response to NT or EGF. In (A), PC3 cells were preincubated with phosphoramidon (300 μM) for 10 min, then stimulated with 20 nM NT or 1 nM EGF for 3 min and Western blotting was performed. Phosphoramidon blocked NT-induced activation of EGFR, AKT and ERK but had no effect on EGF responses. This experiment was performed three times with similar results. In (B), PC3 cells were pretreated for 30 min with phosphoramidon prior to assessing the effects of 20 nM NT and 1 nM EGF on DNA synthesis. Phosphoramidon dose dependently inhibited NT-induced DNA synthesis without altering EGF-induced DNA synthesis. Results were from three experiments performed in quadruplicate. $*p<0.05$; $**p<0.01$ as compared to the control.

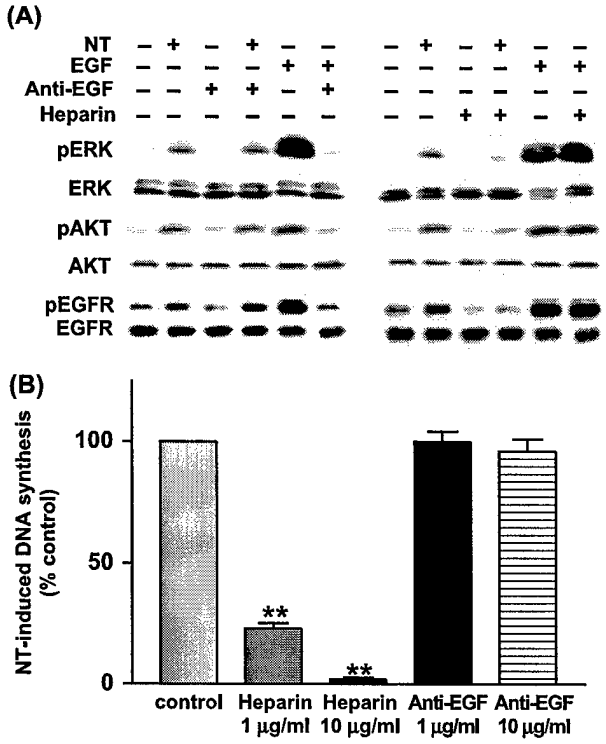

Fig. 6. Effect of heparin and anti-EGF on phosphorylation of ERK, Akt and EGFR (A) and DNA synthesis (B) in response to NT or EGF. In (A), cells were preincubated with heparin (10 µg/ml) or anti-EGF (10 µg/ml) for 10 min and then stimulated with NT (20 nM) or EGF (1 nM) for 3 min. Heparin attenuated NT-induced activation of ERK, Akt and EGFR. In contrast, anti-EGF did not alter these effects although it diminished the response to EGF. This experiment was performed three times with similar results. In (B), PC3 cells were pretreated for 30 min with agents shown, prior to measuring the effect of 20 nM NT on DNA synthesis. Heparin dose-responsively inhibited NT-induced DNA synthesis but anti-EGF did not alter the response. Results were from three experiments performed in quadruplicate. $^{**}p<0.01$ as compared to the control.

stimulation of PLC was not essential for activation of EGFR, Akt and ERK. However, $Ca^{2+}$was required for the downstream DNA synthesis response.

3.6. NT-induced ERK activation and DNA synthesis were PKC-dependent

Enhanced PLC activity is generally associated with the formation of DAG, a PKC activator. To examine the involvement of PKC in the growth responses to NT, cells were treated with PKC inhibitors prior to testing. Staurosporine, bisindolylmaleimide and rottlerin attenuated the effects of NT on activation of EGFR, ERK and Akt (Fig. 9A), as well as on DNA synthesis (Fig. 9B). In addition, down-regulation of PKC by overnight treatment with PMA diminished these responses (Fig. 9A,B). These results indicate that NT-induced EGFR/ERK activation and DNA synthesis are PKC-dependent. The PKC isotypes are likely to be in the conventional (α, β1, β2, γ) or the novel category (δ, ε, η, θ).

3.7. NT decreased EGF binding to PC3 cells

Activation of EGFR leads to receptor desensitization, which can be measured as a decrease in cell surface EGF binding [52]. In some systems, EGFR desensitizes in response to PKC activator PMA [53]. Here, we found that binding of ^{125}I-EGF to PC3 cells in vivo, measured at 37 °C or at 4 °C, was >95% specific. To determine whether NT altered EGF binding, PC3 cells were pretreated with NT for 10 min, and EGF binding was measured. Pretreatment of cells with 20 nM NT decreased EGF binding at 37 °C and at 4 °C by 13 ± 1% and 13 ± 2%, respectively ($n=18$ experiments each; $p<0.05$). Similarly, pretreatment of PC3 cells with PKC activator PMA (0.5 µM) decreased EGF binding at 37 °C and 4°C by 43 ± 3% and 37 ± 4%, respectively ($n=10$ experiments each; $p<0.01$). These results indicated that NT and PKC activator PMA desensitized EGF binding, which was consistent with an ability to activate EGFR.

3.8. Effect of NT on EGF binding was PKC-dependent

Since NT and PMA both activated EGFR and decreased EGF binding, we postulated that their effects were mediated by PKC. To test this hypothesis, we examined the effects of various treatments on NT-induced and PMA-induced EGFR desensitization. As expected, the response to NT and to PMA were both blocked by pretreating cells with AG1478 and with heparin (Table 1), indicating the involvement of HB-EGF and EGFR tyrosine kinase. Both responses were

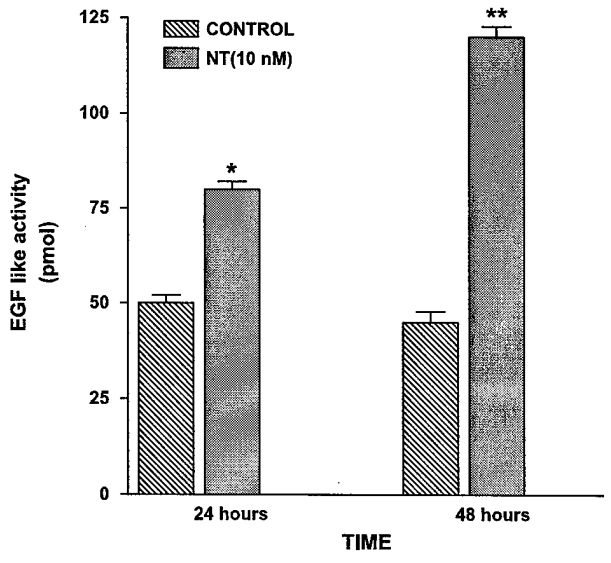

Fig. 7. Effect of NT on the release of EGF-like activity in PC3 cell conditioned media. Quiescent PC3 cells were stimulated with 20 nM NT for 24–48 h. The conditioned media were Sep-Pak concentrated and EGF-like activity was assayed based on its ability to compete with ^{125}I-EGF binding to PC3 cells. NT caused EGF-like activity to accumulate in the media, the levels of which were significantly elevated above control at 24 h (≅60% increase) and 48 h (≅170% increase). Results were from three experiments performed in triplicate. $^{*}p<0.05$; $^{**}p<0.01$ as compared with the basal.

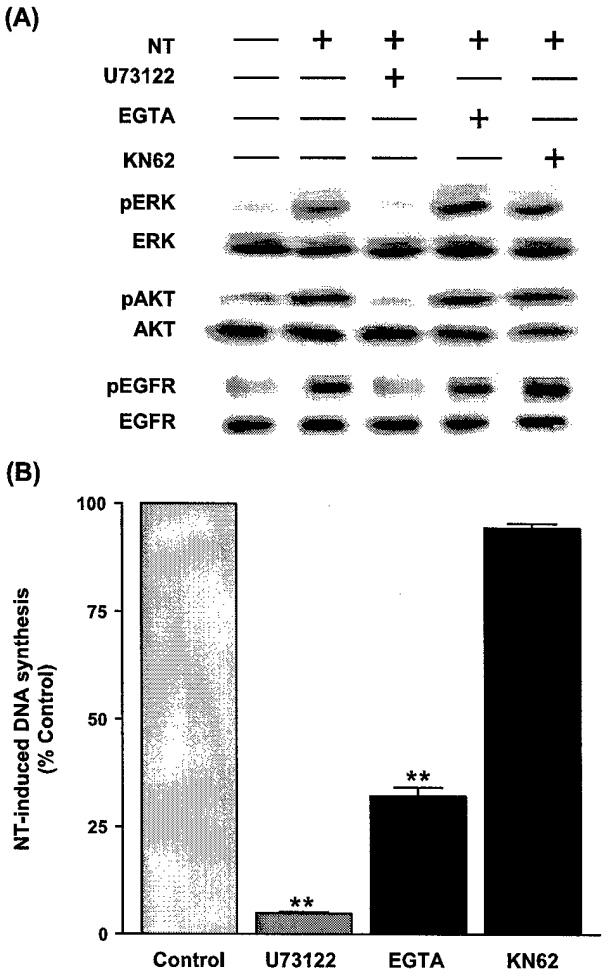

Fig. 8. Effect of PLC, Ca^{2+}/calmodulin kinase inhibitors and Ca^{2+}-chelator in ERK/AKT/EGFR phosphorylation (A) and DNA synthesis (B) in response to NT. In (A), cells were preincubated with PLC inhibitor (U73122, 5 μM) or Ca^{2+}/calmodulin kinase inhibitor (KN62, 10 μM) or Ca^{2+}-chelators (EGTA 0.5 mM in Ca^{+2} free medium) for 10 min and then stimulated with 20 nM NT for 3 min. The effects of NT were blocked by U73122. This experiment was performed three times with similar results. In (B), PC3 cells were pretreated with U73122 (1 μM) or KN62 (10 μM) or EGTA (1.5 mM in Ca^{+2}-containing medium) for 30 min, prior to measuring the effect of 20 nM NT on DNA synthesis. Results were from three experiments performed in quadruplicates. $^{**}p<0.01$ as compared to the control.

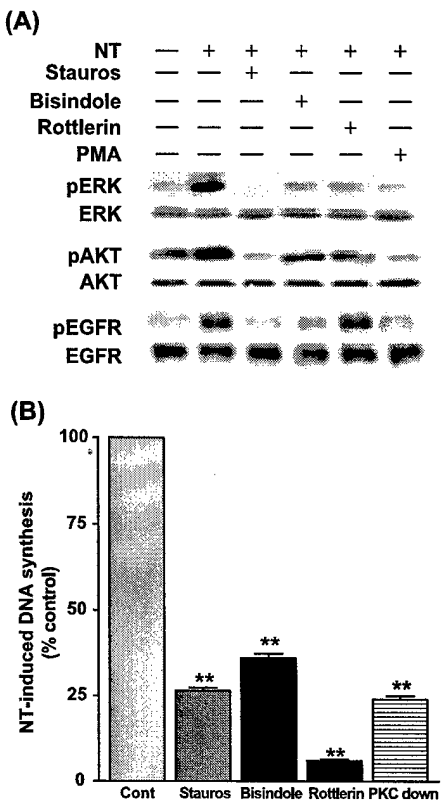

Fig. 9. Effect of PKC inhibitors on phosphorylation of ERK, Akt and EGFR (A) and DNA synthesis (B) in response to NT. In (A), PC3 cells were preincubated with PKC-inhibitors (staurosporine, 2 μM; bisindolylmaleimide I, 10 μM; rottlerin, 10 μM) for 10 min or PKC downregulator PMA (1 μM) for 20 h. After stimulation with NT (20 nM) for 3 min, Western blotting was performed. This experiment was performed three times with similar results. In (B), cells were pretreatment with PKC-inhibitors (staurosporine, 0.1 μM; bisindolylmaleimide, 1 μM; rottlerin, 1 μM) for 30 min or PMA (1 μM) for 20 h prior to measuring the effect of 20 nM NT on DNA synthesis. Results were from three experiments performed in quadruplicate. $^{*}p<0.01$ as compared to the control.

inhibited by downregulating PKC (via an overnight treatment of cells with PMA), and by pretreating cells with PKC inhibitors staurosporine, bisindolylmaleimide and rottlerin (Table 1). Taken together, these results supported the idea that the NT-induced activation (and subsequent desensitization) of EGFR was ligand dependent and involved PKC, most likely novel isotypes (δ, ε, η, θ).

3.9. Role of PI3-kinase in NT-induced growth effects

To examine the relationships amongst PI3-kinase, ERK and DNA synthesis in response to NT, cells were pretreated

Table 1
Effects of various treatments on NT-induced and PMA-induced desensitization of EGF binding

Treatment[a]	EGF binding desensitization response (%)[b]	
	NT-induced	PMA-induced
Vehicle Control	100	100
Heparin (3 μg/ml)	40 ± 8*	58 ± 14*
AG1478 (1 μM)	16 ± 4**	33 ± 5*
PKC downregulation	13 ± 4**	23 ± 5**
Staurosporine (1 μM)	33 ± 5*	32 ± 5*
Bisindolemaleimide (10 μM)	33 ± 5*	43 ± 6*
Rottlerin (10 μM)	42 ± 5*	78 ± 8

[a] PC3 cells in 24-well plates were pretreated for 10 min with the agents indicated. Then NT (20 nM) or PMA (0.5 μM) was added for 10 min, and EGF binding was measured as described in Section 2.

[b] For the control condition, NT and PMA reduced EGF binding by 15 ± 3% and 47 ± 5%, respectively. Given is the mean response (± S.E.M.) determined in three to four experiments.

* Statistical analysis indicated $p<0.05$ relative to control.

** Statistical analysis indicated $p<0.01$ relative to control.

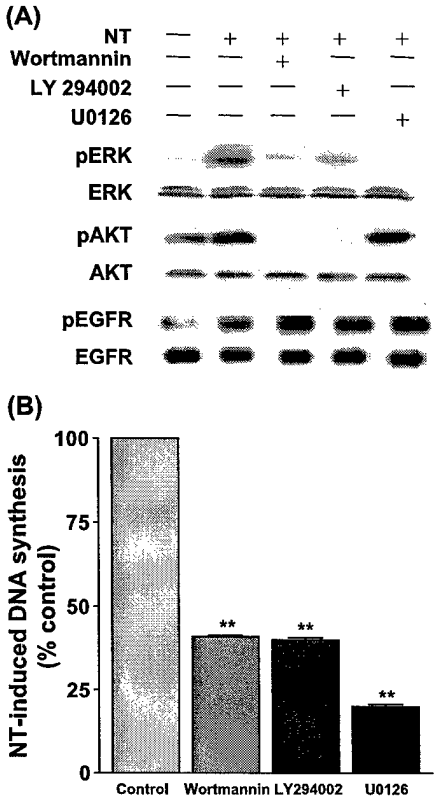

Fig. 10. Effect of MEK1/2 and PI3-kinase inhibitors in ERK/AKT/EGFR phosphorylation (A) and DNA synthesis (B) in response to NT. In (A), cells were preincubated with MEK1/2 inhibitor (U0126, 10 μM;) or PI3-kinase inhibitors (wortmannin, 2.5 μM; LY 294002, 10 μM) for 10 min and then stimulated with NT (20 nM) for 3 min. These experiments were performed three times with similar results. In (B), cells were pretreated with MEK1/2 inhibitor (U0126, 1 μM) or PI3-kinase inhibitors (wortmannin, 0.5 μM; LY 294002 1 μM) for 30 min followed by 24-h NT stimulation and 3-h pulse of [^{3}H]thymidine. Results were from three experiments performed in quadruplicates. *$p < 0.05$ as compared with the control.

with specific inhibitors prior to testing. PI3-kinase inhibitors (wortmannin, LY294002) as well as MEK inhibitor (U0126) attenuated the effects of NT on ERK activation (Fig. 10A) and DNA synthesis (Fig. 10B); however, they did not inhibit the effect of NT on EGFR phosphorylation (Fig. 10A). These results indicated that PI3-kinase and MEK were downstream of EGFR and were essential for NT-induced ERK activation and DNA synthesis.

4. Discussion

NT regulates the growth of gastrointestinal tissues [19] and stimulates proliferation of cancer cells derived from lung, colon, pancreas and prostate [16,21–23]. To further elucidate the signal transduction mechanisms involved in the trophic effects of NT, the present study used prostate cancer epithelial PC3 cells, which express high levels of NTR1 and display enhanced growth in response to NT [16]. The primary focus was to examine the involvement of EGFR, MAP-kinases (ERK, SAPK/JNK and p38) and PI3 kinase in the mitogenic effect of NT. Our results indicated that NT-induced mitogenesis involved PKC-dependent, ligand-mediated transactivation of EGFR, which led to stimulation of the Raf–MEK–ERK pathway in a PI3 kinase-dependent manner.

GPCR agonists can employ multiple distinct pathways to activate MAP-kinases and stimulate cell growth and survival responses [31–33]. One mechanism, demonstrated for both Gi- and Gq-coupled receptors, involves transactivation of the receptor tyrosine kinase EGFR [34–37]. To determine the role of EGFR in mediating growth responses to NT, we tested specific tyrosine-kinase inhibitors for the ability to block NT-induced activation (phosphorylation) of EGFR and ERK, and to inhibit DNA synthesis. Finding that AG1478 and PD153035 inhibited each of these responses to NT, we concluded that EGFR was an essential participant in the mechanism. That the activation of EGFR was causal, not just permissive, for NT-induced DNA synthesis was indicated by the time-course, dose response and inhibitor dependence of these effects. In addition, NT-induced DNA synthesis and EGF-induced DNA synthesis were non-additive and both displayed a similar sensitivity to contact inhibition (Ref. [54] and Hassan, unpublished data).

Accumulating evidence indicates that EGFR is involved in signal transduction for many growth-promoting GPCR agonists, including lysophosphatidic acid, endothelin, thrombin, bradykinin, angiotensin II, bombesin and carbachol [31–36]. The activation of EGFR is often mediated by liberation of EGF-like ligands [34–36], but this is not always the case [37–39]. A variety of mechanisms have been proposed, some involving Ca^{2+} [55], non-receptor tyrosine kinases such as PYK2 [56,57], reactive oxygen species [58] and metalloproteinases [59,60]. In PC3 cells, the present study is the first to report on the involvement of EGFR in the mitogenic effect of NT; however, bombesin [36], bradykinin [14] and lysophosphatidic acid [7] have been shown to activate EGFR. Our findings are in keeping with those for bombesin, which implicate HB-EGF and a metalloproteinase in the mechanism.

Based on the rapid onset of EGFR phosphorylation and the failure to detect EGF-like ligands in conditioned media, workers in other systems suggested that transactivation of EGFR could be ligand-independent [35,55]. Evidence for a direct activation via a multireceptor complex has been found in some systems [61]. In contrast, our results support the involvement of EGF-like ligand(s) in the response to NT. We demonstrated the release of EGF-like material in conditioned media at 24–48 h after addition of NT using a cell-based EGFR binding assay. Many EGF-related substances—including HB-EGF, TGFα, amphiregulin and epiregulin—can bind to EGFR and induce mitogenesis [8]. The expression of each of these substances is enhanced 10–100 fold in PC3 cells and this may relate to the androgen-independent growth of these cells [62]. These growth factors are synthesized as transmembrane precursors that are cleaved to yield the soluble, active ligands [34–36]. A distinguishing feature of

HB-EGF and amphiregulin is their ability to bind to heparin [8,62]. In the present study, heparin (but not neutralizing anti-EGF) blocked NT-induced EGFR/ERK activation and DNA synthesis, indicating an important role for HB-EGF or amphiregulin in mediating the response to NT. On the other hand, anti-EGF (but not heparin) blocked the effects of EGF, demonstrating the specificity of these reagents.

Activation of EGFR normally leads to internalization of cell-surface EGFR and downregulation/desensitization of EGF binding. In other systems, these responses can be initiated by GPCR agonists and can be mimicked by activation of PKC [37,54]. In the present study, NT was shown to downregulate EGF binding in PC3 cells and this response was antagonized by heparin and by inhibitors of PKC. These results are consistent with the involvement of HB-EGF or amphiregulin in the transactivation of EGFR by NT, and suggest that liberation of the EGF-like ligand is PKC-dependent. In keeping with this idea, PKC-activator PMA reproduced the effects of NT, activating EGFR and the ERK pathway, and causing downregulation of EGF binding. It is now clear from numerous reports that at least three EGF-like ligands (HB-EGF, amphiregulin and TGFα) can participate in EGFR transactivation [34]. The general mechanism seems to involve PKC-mediated activation of metalloproteases, leading to a shedding of EGF-like ligands from the cell surface [35,36]. In the present study, our finding that phosphoramidon prevented NT-induced EGFR/ERK activation and DNA synthesis is consistent with the involvement of a similar mechanism in this case.

Mitogenic signaling via EGFR is mediated largely through the Raf–MEK–ERK pathway, which carries the signal from the cell surface to the nucleus, culminating in altered gene expression, DNA synthesis and cell proliferation [10–12]. In the present study, we demonstrated that NT utilized EGFR to induce ERK activation, which led to enhanced DNA synthesis and cell proliferation. DNA synthesis was enhanced ≅2.0-fold by maximal doses of NT and ≅2.2-fold by EGF. Their combined effect, ≅2.5-fold enhancement, was less than additive. This suggested that key step(s) in their mechanistic pathways were shared. Although EGF was far more effective than NT in stimulating ERK phosphorylation, this might indicate that ERK phosphorylation and DNA synthesis were not linearly related. Nevertheless, MEK inhibitor U0126 abolished the stimulatory effect of NT on ERK phosphorylation and DNA synthesis, indicating that MEK was essential for both responses. Collectively, these findings establish the critical involvement of EGFR and ERK in the mitogenic effect of NT. However, the signaling mechanism employed by NTR1 and the precise points of entry into the ERK pathway require further scrutiny.

A common feature of NT signaling in transfected cells and cancer cell lines expressing NTR1 is activation of PLC, resulting in enhanced inositol phospholipid hydrolysis and liberation of internal Ca^{2+}stores [63]. Although liberation of DAG and activation of PKC are expected to follow the stimulation of PLC, these are not always observed with NT [64]. With this background, we determined the involvement of PLC in NT-induced EGFR/ERK activation and DNA synthesis. Finding that the PLC inhibitor U71322, as well as the PI4/PI5-kinase inhibitor quercetin, attenuated these NT-induced effects, we concluded that PLC and inositol phosphates were involved in the mitogenic response to NT. An elevation in cytosolic [Ca^{2+}] normally follows activation of PLC. To assess the importance of Ca^{2+}, we tested Ca^{2+}-chelator (EGTA) and Ca^{2+}/calmodulin inhibitor (KN62) for ability to alter the effects of NT. Activation of EGFR, ERK and Akt in response to NT was not diminished by EGTA or KN62. Although NT-induced DNA synthesis was inhibited by EGTA, this most likely reflected a general effect of Ca^{2+}-withdrawal on cell health [65].

Activation of PLC can influence PKC activity via the generation of DAG. Since PKC activators can stimulate the ERK pathway [35], the role of PKC in NT-induced EGFR/ERK activation and DNA synthesis was examined. These effects of NT were attenuated by PKC inhibitors bisinolylmaleimide, staurosporine and rottlerin, as well as by downregulating PKC expression. Although this indicates an involvement of PKC in the mitogenic response to NT, it is not yet clear where PKC acts and whether it plays causal or permissive role(s). Since PKC activator PMA can initiate protease-mediated liberation of HB-EGF [36,59], this is one possible causal role. PKC can also influence Raf activity [42,66] and, depending on the level of basal activity, this could be permissive. Our finding that PMA reproduced the effects of NT on EGFR, ERK and Akt argues for a causal role for PKC as mediator of these actions. However, the fact that PMA inhibited (rather than stimulated) DNA synthesis is not consistent. Possible explanations include (a) that the stimulatory effect of NT on DNA synthesis involves activation of a select subset of PKCs, while blanket activation is inhibitory; (b) that NT (unlike PMA) activates PKC without causing subsequent downregulation; (c) that NT does not activate PKC but its effects require constitutive PKC activity. In preliminary studies, we found that PC3 cells express PKCα, PKCβ1, PKCδ and PKCε. However, these PKC isotypes were not activated by a 5 min exposure to NT (as indicated by phosphorylation or translocation to membrane), nor were they downregulated 24 h later. In addition, we found that ERK activation in response to NT was sensitive to the novel PKC inhibitor Go-6983, while ERK activation in response to EGF was sensitive to the conventional PKC inhibitor Go-6976. Although we cannot draw a definitive conclusion yet, our results are most consistent with PKC playing causal and permissive roles in NT-induced mitogenesis. While a novel PKC is likely to mediate the liberation of HB-EGF and the activation of EGFR, conventional PKC(s) may regulate the activation of Raf in a permissive manner.

The PI3 kinase pathway participates in the growth, survival and differentiation of mammalian cells and studies have shown that its effects on cell growth are cell-type and

stimulus dependent [48,49]. The results presented here indicate that PI3 kinase is involved in NT-induced ERK activation and DNA synthesis. NT not only caused a dose- and time-dependent activation of Akt, but inhibitors of PI3 kinase (wortmannin and LY-294002) blocked NT-stimulated ERK activation and DNA synthesis. Therefore, it is possible that the PI3 plays an impotent role in transmitting the growth signal of NT in PC3 cells. In some systems, PI3 kinase is thought to assist in the activation of Raf by facilitating its translocation to membranes [37,43].

Considerable evidence argues for a correlation between aberrant GPCR signaling and the development and progression of human cancers [31]. In regards to NT, blood levels of this peptide increase with high fat intake, which is associated with a higher incidence of prostate cancer. Here, we demonstrate that NT can promote PC3 cell growth by transactivating EGFR and stimulating the Raf–MEK–ERK pathway. In late-stage cancers, the potential for signaling along this pathway is enhanced by the increased expression of NTR1, EGFR and ERK. Not only is the ERK level elevated but ERK phosphorylation is most prevalent in late-stage tumors and in recurrent tumors. Coupled with the present results, these findings suggest that NT might contribute to androgen-independent growth of cancer cells and they provide a possible explanation for the effects of high fat diets on cancer incidence.

Acknowledgements

We thank Sheryl Dooley for laboratory help. This work was supported by Department of Defense (DOD) Grant DAMD 17-00-1-0528, although the opinions expressed are not necessarily those of the DOD.

References

[1] Boring CC, Squires TS, Tong T. Cancer statistics. CA Cancer J Clin 1993;43(1):7–26.
[2] Carter HB, Isaacs JT. Overview of hormonal therapy for prostate cancer. Prog Clin Biol Res 1990;350:129–40.
[3] Jones HE, Eaton CL, Barrow D, Dutkowski CM, Gee JM, Griffiths K. Comparative studies of the mitogenic effects of epidermal growth factor and transforming growth factor-alpha and the expression of various growth factors in neoplastic and non-neoplastic prostatic cell lines. Prostate 1997;30:219–31.
[4] Eaton CL, Davies P, Phillips ME. Growth factor involvement and oncogene expression in prostatic tumours. J Steroid Biochem 1988; 30(1–6):341–5.
[5] Gioeli D, Mandell JW, Petroni GR, Frierson Jr HF, Weber MJ. Activation of mitogen activated protein kinase associated with prostate cancer progression. Cancer Res 1999;59:279–84.
[6] Price DT, Della Rocca GJ, Guo C, Ballo MS, Schwinn DA, Luttrell LM. Activation of extracellular signal-regulated kinase in human prostate cancer. J Urol 1999;162:1537–42.
[7] Kue PF, Taub JS, Barki-Harrington L, Polakiewicz RD, Ullrich A, Daaka Y. Lysophosphatidic acid-regulated mitogenic ERK signaling in androgen-insensitive prostate cancer PC3 cells. Int J Cancer 2002: 572–9.
[8] Massague J, Pandiella A. Membrane-anchored growth factors. Annu Rev Biochem 1993;62:515–41.
[9] Adam RM, Kim J, Lin J, Orsola A, Zhuang L, Rice DC, et al. Heparin-binding epidermal growth factor-like growth factor stimulates androgen-independent prostate tumor growth and antagonizes androgen receptor function. Endocrinology 2002;143(12): 4599–608.
[10] Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79:143–80.
[11] Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J. Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol 2002;4(8):556–64.
[12] Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J 1999;18(3):664–74.
[13] Falkmer S, Askensten L, Grimelius L, Abrahamsson PA. Cytochemical markers and DNA content of neuroendocrine cells in carcinoma of the prostate gland during tumour progression. Acta Histochem 1990;38:127–32 [Suppl].
[14] Barki-Harrington L, Daaka Y. Bradykinin induced mitogenesis of androgen independent prostate cancer cells. J Urol 2001;165:2121–5.
[15] Segal I, Powers S, Huntley B, Powis G, Pittelkow M, Maihle NJ. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. Proc Natl Acad Sci, U S A 1994;91:4673–7.
[16] Seethalakshmi L, Mitra SP, Dobner PR, Menon M, Caraaway RE. Neurotensin receptor expression in prostate cancer cell line and growth effect of NT at physiological concentrations. Prostate 1997; 31:183–92.
[17] Carraway RE, Leeman SE. Characterization of radioimmunoassayable neurotensin in the rat: its differential distribution in the central nervous system, small intestine and stomach. J Biol Chem 1976;251: 7045–52.
[18] Reinecke M. Neurotensin: immunohistochemical localization in central and peripheral nervous system and in endocrine cells and its functional role as neurotransmitter and endocrine hormone. Prog Histochem Cytochem 1985;16:1–175.
[19] Wood JG, Hoang HD, Bussjaeger LJ, Solomon TE. Neurotensin stimulates growth of the small intestine in rats. Am J Physiol 1988; 255:813–7.
[20] Tatsuta M, Iishi H, Baba M, Taniguchi H. Promotion by neurotensin of gastric carcinogenesis induced by *N*-methyl-*N'* -nitro-*N*-nitrosoguanidine in Wistar rats. Cancer Res 1989;49:843–6.
[21] Ishizuka J, Townsend CM, Thompson JC. Neurotensin regulates growth of human pancreatic cancer. Ann Surg 1993;217:439–46.
[22] Maoret JJ, Anini Y, Rouyer-Fessard C, Gully D, Laburthe M. Neurotensin and a non-peptide neurotensin receptor antagonist control human colon cancer cell growth in cell culture and in cell xenografted into nude mice. Int J Cancer 1999;80:448–54.
[23] Davis TP, Burgess HS, Crowell S, Moody TW, Gulling-Berglund A, Liu RH. β-Endorphin and neurotensin stimulate in vitro clonal growth of human SCLC cells. Eur J Pharmacol 1989;161:283–5.
[24] Rosell S, Rokaeus A. The effects of ingestion of amino acids, glucose and fat on circulating neurotensin-like immunoreactivity (NTLI) in man. Acta Physiol Scand 1979;107:263–7.
[25] Ferris CF. Neurotensin. Handbook of Physiology. The Gastrointestinal System. Bethesda, MD. Am Physiol Soc, vol. 6; 1989. p. 559–86.
[26] Giovanucci EJ, Rimm EB, Colditz GA. A prospective study of dietary fat and risk of prostate cancer. J Natl Cancer Inst 1993;85:1571–9.
[27] Elek J, Pinzon W, Park KH, Narayanan R. Relevant genomics of neurotensin receptor in cancer. Anticancer Res 2000;20:53–8.
[28] Vincent JP, Mazella J, Kitabgi P. Neurotensin and neurotensin receptors. Trends Pharmacol Sci 1999;20(7):302–9.
[29] Hasagawa K, Siddhartha K, Carr BI. Stimulation of hepatocyte DNA synthesis by neurotensin. J Cell Physiol 1994;158:215–22.

[30] Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 2001;20:1532–9.
[31] Marinissen MJ, Gutkind JS. G protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 2001;22:368–76.
[32] Della Rocca GJ, vam Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. J Biol Chem 1997;272: 19125–32.
[33] Luttrell LM, Daaka Y, Lefkowitz RJ. Regulation of tyrosine kinase cascades by G protein-coupled receptors. Curr Opin Cell Biol 1999; 11:177–83.
[34] Gschwind A, Hart S, Fischer OM, Ullrich A. TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. EMBO J 2003;22:2411–21.
[35] Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signaling by G protein-coupled receptors. Nature 1996;379:557–60.
[36] Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 1999; 402(6764):884–8.
[37] Andresen BT, Linnoila JJ, Jackson EK, Romero GG. Role of EGFR transactivation in angiotensin II signaling to extracellular regulated kinase in preglomerular smooth muscle cells. Hypertension 2003;41: 781–6.
[38] Wang H, Ubl JJ, Stricker R, Reiser G. Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. Am J Physiol Cell Physiol 2002;283(5):C1351–64.
[39] Seta K, Sadoshima J. Phosphorylation of tyrosine 319 of the angiotensin II type 1 receptor mediates angiotensin II-induced transactivation of the epidermal growth factor receptor. J Biol Chem 2000;278:9019–26.
[40] Tice DA, Biscardi JS, Nickles AL, Parsons SJ. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. Proc Natl Acad Sci, U S A 1999;96:1415–20.
[41] Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. A role for Pyk2 and Src in linking G protein-coupled receptors with MAP kinase activation. Nature 1997;383:547–9.
[42] Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, et al. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 1993;364(6434):249–52.
[43] Rizzo MA, Shome K, Watkins SC, Romero G. The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. J Biol Chem 2000;275:23911–8.
[44] Newton AC. Regulation of protein kinase C. Curr Opin Cell Biol 1997;9:161–7.
[45] Ehlers RA, Zhang Y, Hellmich MR, Evers BM. Neurotensin-mediated activation of MAPK pathways and AP-1 binding in the human pancreatic cancer cell line, MIA PaCa-2. Biochem Biophys Res Commun 2000;269(3):704–8.
[46] Mitra SP, Carraway RE. Synergistic effects of neurotensin and beta-adrenergic agonist on 3, 5-cyclic adenosine monophosphate accumulation and DNA synthesis in prostate cancer PC3 cells. Biochem Pharmacol 1999;57(12):1391–7.
[47] Carraway RE, Gui X, Cochrane DE. Ca^{2+}-channel blockers enhance neurotensin (NT) binding and inhibit NT-induced inositol phosphate formation in prostate cancer PC3 cells. J Pharmacol Exp Ther 2003;307:640–50.
[48] Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2(7):489–501.
[49] Roche S, Koegl M, Courtneidge SA. The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. Proc Natl Acad Sci U S A 1994 (Sep 13); 91(19):9185–9.
[50] Higashiyama S, Abraham JA, Klagsbrun M. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. J Cell Biol 1993;122:933–40.
[51] Singhal RI, Prajda N, Yeh YA, Weber G. 1-Phosphatidylinositol 4-phosphate 5-kinase (EC 2.7.1.68): A proliferation- and malignancy-linked signal transduction enzyme. Cancer Res 1994;54:5574–8.
[52] Grewal JS, Luttrell LM, Raymond JR. G protein-coupled receptors desensitize and down-regulate epidermal growth factor receptors in renal mesangial cells. J Biol Chem 2001;276(29):27335–44.
[53] Goishi K, Higashiyama S, Klagsbrun M, Nakano N, Umata T, Ishikawa M, et al. Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. Mol Biol Cell 1995; 6(8):967–80.
[54] Iwamoto R, Handa Km, Mekada E. Contact-dependent growth inhibition and apoptosis of epidermal growth factor (EGF) receptor-expressing cells by the membrane-anchored form of heparin-binding EGF-like growth factor. J Biol Chem 1999;274(36):25906–12.
[55] Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsunomiya H, et al. Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. J Biol Chem 1998;273:8890–6.
[56] Eguchi S, Iwasaki H, Inagami T, Numaguchi K, Yamakawa T, Motley ED, et al. Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. Hypertension 1999;33:201–6.
[57] Shah BH, Catt KJ. Calcium-independent activation of extracellularly regulated kinases 1 and 2 by angiotensin II in hepatic C9 cells: roles of protein kinase Cδ, Src/proline-rich tyrosine kinase 2 and epidermal growth factor receptor treas-activation. Mol Pharmacol 2001;61: 343–51.
[58] Ushio-Fukai M, Griendling KK, Becker PL, Hilenski L, Halleran S, Alexander RW. Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2001;21(4):489–95.
[59] Dethlefsen SM, Raab G, Moses MA, Adam RM, Klagsbrun M, Freeman MR. Extracellular calcium influx stimulates metalloproteinase cleavage and secretion of heparin-binding EGF-like growth factor independently of protein kinase C. J Cell Biochem 1998;69: 143–53.
[60] Uchiyama-Tanaka Y, Matsubara H, Nozawa Y, Murasawa S, Mori Y, Kosaki A, et al. Angiotensin II signaling and HB-EGF sheding via metalloproteinase in glomerular mesangial cells. Kidney Int 2001;60: 2153–63.
[61] Maudsley S, Pierce KL, Zamah M, Miller WE, Ahn S, Daaka Y, et al. The β2-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. J Biol Chem 2000;275:9572–80.
[62] Torring N, Jorgensen PE, Sorensen BS, Nexo E. Increased expression of heparin binding EGF (HB-EGF), amphiregulin, TGFα and epiregulin in androgen-independent prostate cancer cell lines. Anticancer Res 2000;20:91–6.
[63] Hermans E, Maloteaux JM. Mechanisms of regulation of neurotensin receptors. Pharmacol Ther 1998;79:89–104.
[64] Bozou JC, Rochet N, Magnaldo I, Kitabgi P, Vincent JP. Neurotensin stimulates inositol trisphosphate-mediated calcium mobilization but not protein kinase C activation in HT29 cells. Biochem J 1989;264: 871–8.
[65] Skryma R, Mariot P, Bourhis XL, Coppenolle FV, Shuba Y, Vander Abeele F, et al. Store depletion and stor-operated Ca2+ current in human prostate cancer LNCaP cells: involvement in apoptosis. J Physiol 2000;527:71–83.
[66] Corbit KC, Trakul N, Eves EM, Diaz B, Marshall M, Rosner MR. Acativation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. J Biol Chem 2003;278: 13061–8.